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**Influence of ApoE polymorphism  
on synaptic morphometry during aging  
in the dentate gyrus  
of ApoE knockout and human ApoE transgenic mice**

by Karine Cambon

A thesis submitted in partial satisfaction for the degree of  
Doctor of Philosophy

Submitted March 2000

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## Abbreviations

Ach	acetylcholine
AchE	acetylcholinesterase
AD	Alzheimer's disease
ApoE	Apolipoprotein E
APP	amyloid precursor protein
AZ	apposition zone
A $\beta$	$\beta$ -amyloid protein
ChAT	choline acetyl transferase
CNS	central nervous system
CSF	cerebrospinal fluid
DG	dentate gyrus
EC	entorhinal cortex
ECL	entorhinal cortex lesion
EM	electron microscopy
hApoE	human apolipoprotein E
HDL	high density lipoprotein
HSPG	heparan sulfate proteoglycan
ICC	immunocytochemistry
IML	inner molecular layer
KO	knock-out
LDL	low density lipoprotein
LRP	LDL receptor related protein
MAP2c	microtubule associated protein
Mean AZ/syn	mean AZ area per synapse
ML	molecular layer
MML	middle molecular layer
MRI	magnetic resonance imaging
NFT	neurofibrillary tangle
NT	neuropil thread
OML	outer molecular layer
PHF	paired helical filament
PP	perforant path
PSD	post synaptic density
SEM	standard error of the mean
Syn/nrn	synapse per neuron ratio
VLDL	very low density lipoprotein
WT	wild type

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## Abstract

Of the three forms of human Apolipoprotein E (ApoE2, ApoE3 and ApoE4), the  $\epsilon 4$  allele coding for ApoE4 is associated with a higher risk of developing Alzheimer's disease (AD) and an earlier age of onset, whereas  $\epsilon 2$  may be protective. The mechanisms underlying such influences are still unclear.

This thesis has investigated the influence of ApoE polymorphism on structural synaptic parameters in the middle molecular layer (MML) of the dentate gyrus of wild type (WT), ApoE knockout (KO) and human ApoE (hApoE) transgenic mice, from 6 to 24 months of age using unbiased stereological methods at the EM level. In hApoE4 mice, there was a 34% decrease in the synapse per neuron ratio (Syn/nrn) accompanied by a 22% increase in the mean apposition zone area (mAZA) during aging. This pattern resembles closely the synaptic changes occurring early in AD, which have been correlated to the first symptoms of memory loss in humans. In contrast, there was no such synaptic loss in hApoE2, ApoE KO and WT mice. At old age, hApoE4 mice had the lowest syn/nrn but their mAZA was comparable to that of other groups. These data appear to be consistent with the view that possession of  $\epsilon 4$  is deleterious to cognitive functions in the elderly and AD patients. Notably, aged ApoE KO mice did not show any sign of synaptic degeneration, suggesting the involvement of other proteins to compensate for the lack of ApoE. At 18 months old, hApoE2 mice have a greater hippocampal volume and display the highest syn/nrn and glutamate immunogold labelling in presynaptic boutons and dendrites of the MML, compared to hApoE3 and hApoE4 mice. In AD patients, such effects of the  $\epsilon 2$  allele may act as a synaptic 'reservoir' and delay the onset of AD symptoms.

Thus, these lines of hApoE transgenic mice could provide a good basis for the future production of multiple transgenic mice in which to model AD pathogenesis.



## **Chapter 1   Introduction**

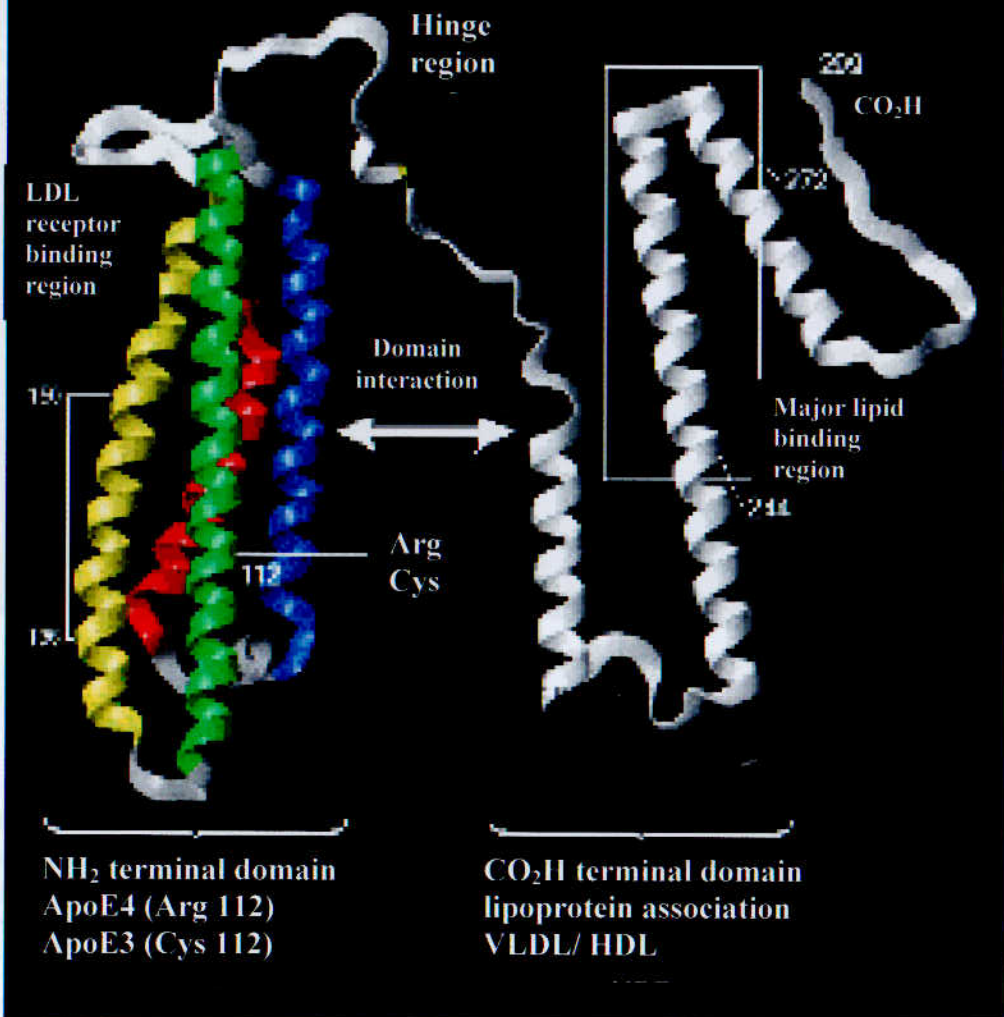
## **1.1 General introduction**

Twenty six years ago, ApoE was first described by Shore and Shore (Shore & Shore 1973) as a component of very low density lipoprotein (VLDL) and initial interest focussed on its role in cholesterol-induced hyperlipidemia (Mahley 1983). Along with the discovery of the central role of ApoE in lipoprotein metabolism, additional roles have since been found in areas such as neurobiology (Mahley 1988). The structure of ApoE and its expression in the brain are described in sections 1.1.1 and 1.1.2. The findings that support its involvement in various areas of neurobiology, with a particular emphasis on neuronal injury and Alzheimer's disease (AD) are explored in sections 1.1.3 to 1.2. The last section describes the current ApoE transgenic models used to study the mechanisms by which ApoE polymorphism influences the risk and age of onset of AD, and the outcome of head injury.

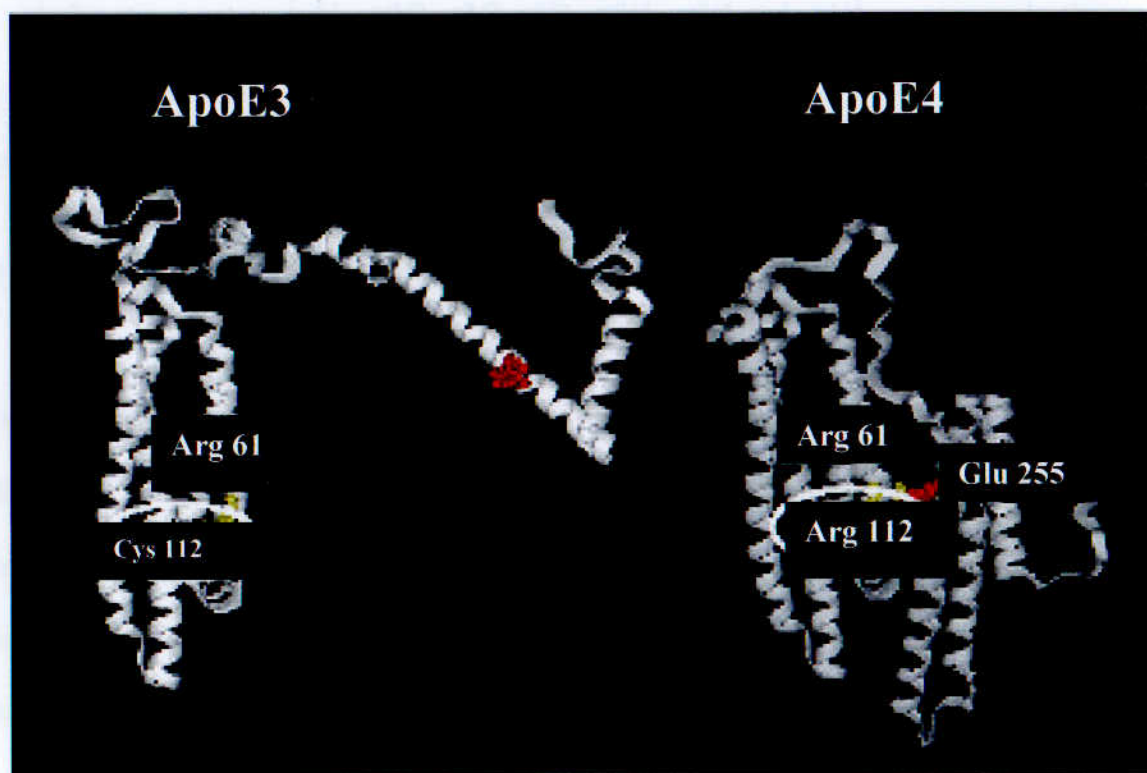
### **1.1.1 ApoE structure.**

Apolipoprotein E (ApoE) is a 299- amino acid plasma glycoprotein with multiple biological properties. In humans, there are 3 isoforms of ApoE, named ApoE2, E3 and E4, which are encoded by 3 different alleles  $\epsilon 2$ ,  $\epsilon 3$  and  $\epsilon 4$  on chromosome 19. The 3 different ApoE isoforms differ by a single amino acid substitution (Figure 1.1 and Figure 1.2): ApoE2 has cysteine residues on positions 112 and 158, whereas ApoE3 has an arginine at position 158 and ApoE4 has an arginine at both positions (Mahley 1988). In rodents however, only one ApoE isoform has been identified and it is most similar to the human ApoE4 protein (Rajavashisth et al. 1985). 3-dimensional structural studies of the different isoforms have shown that arginine in position 112 confers a typical conformation to the ApoE4 protein and distinguishes it from ApoE2 and ApoE3 (Figure 1.2). This conformational difference is thought to underlie the differential effects of the ApoE isoforms on the CNS structure and function (Mahley & Huang 1999).

## ApoE Domain Structure



*Figure 1.1 The 2-domain structure of ApoE. As determined by x-ray crystallography, the amino terminal domain is folded in a 4-helix bundle motif, whereas the structure of the carboxyl terminal domain is still unclear and depicted as a series of  $\alpha$ -helices. The receptor binding region is located in the amino terminal domain on helix 4, while the carboxyl terminal domain contains the major lipid-binding elements of the protein (adapted from K.H. Weisgraber, Gladstone Institute web site).*



*Figure 1.2 The structure of ApoE3 and ApoE4 as determined by x-ray crystallography. Amino acid differences at position 112 distinguish ApoE3 (Cys) and ApoE4 (Arg). The interaction of Arg 61(amino terminal domain) and Glu 255 (carboxy terminal domain) modifies ApoE4 domain interaction and is responsible for the very low density lipoprotein (VLDL) binding preference of ApoE4 in plasma (adapted from K.H. Weisgraber, Gladstone Institute web site).*

Every human individual carries 2 copies of the ApoE gene, one from each parent. Thus 6 combinations are possible:  $\epsilon 2/2$ ,  $\epsilon 2/3$ ,  $\epsilon 3/3$ ,  $\epsilon 2/4$ ,  $\epsilon 3/4$  and  $\epsilon 4/4$  genotypes. In the general population, the most common allele is Apo $\epsilon 3$  (78%), followed by  $\epsilon 4$  (15%) and  $\epsilon 2$  (7%) (Mahley 1988, Zannis et al. 1993).

### **1.1.2 ApoE expression in the nervous system.**

In 1985, Elshourbagy et al (Elshourbagy et al. 1985) showed that ApoE was produced in abundance in the brain. ApoE was then shown to be associated with lipoproteins, and described as the principal lipid transport vehicle in cerebrospinal fluid (Pitas et al. 1987). Moreover, in peripheral nerve injury experiments, ApoE was induced at high concentration and appeared to play a key role in repair by redistributing lipids to regenerating axons and to Schwann cells during remyelination (Boyles et al. 1989, Ignatius et al. 1986). Taken together, these findings were the first indications that ApoE had a potential role in the central nervous system.

In plasma and CSF, ApoE is associated with lipoproteins (Figure 1.3). These are macromolecular complexes generally composed of a phospholipid and free cholesterol shell surrounding a triglyceride and cholesteryl ester core in plasma, without triglyceride in CSF, and without either of these two molecules when secreted by astrocytes (Fagan et al. 1999, LaDu et al. 1998, Mahley 1988). Apolipoproteins such as ApoE stabilise the surface of lipoproteins, and serve as cofactors for enzymatic reactions and as ligands for cell surface receptors (Mahley 1988).

Several studies have now shown the presence of immunoreactive ApoE in both neurons and glial cells in normal human and primate brain whereas it is essentially restricted to glial cells in normal rodent brain (Boyles et al. 1985, Diedrich et al. 1991, Han et al. 1994a (Han et al. 1994b, Ignatius et al. 1986, Poirier et al. 1991, Metzger et al. 1996, Rebeck et al.

1993, Xu et al. 1996, Xu et al. 1999a, Zarow & Victoroff 1998). At the ultrastructural level, glial and neuronal ApoE immunoreactivity is found in clumps in the cytoplasm and seems to be associated with the membrane of the endoplasmic reticulum or other organelles and cellular structures like mitochondria. Whereas astrocytes normally display a strong immunostaining from the soma to distal processes, only the cell body of neurons and not their axons or distal dendrites, is immunoreactive. No staining of pre- or post-synaptic profiles is observed (Han et al. 1994a) although ApoE has been detected at the neuromuscular junction in rodents and humans (Akaaboune et al. 1994).

In the brain, 4 main ApoE receptors have been identified (Table 1). Members of the low-density lipoprotein (LDL) receptor family, such as the LDL receptor and the LDL receptor-related protein (LRP) can mediate the uptake of ApoE lipoprotein enriched particles into cells (Krieger & Herz 1994, Mahley 1988). In the normal human brain, granule cells of the DG and pyramidal neurons of other hippocampal subfields are strongly immunostained for the LRP receptor. Whereas astrocytes are lightly immunostained in the normal brain, activated astrocytes in the brain of AD patients possess a high density of LRP receptors (Rebeck et al. 1993, Rebeck et al. 1995). In contrast, anti-LDL antibodies weakly stain astrocytes and the neuropil both in normal and in AD brain (Rebeck et al. 1993). The very low density lipoprotein (VLDL) receptor and the ApoE receptor 2 (ApoER2) have been localised in pyramidal neurons and in granule cells (Kim et al. 1996, Christie et al. 1996, Page et al. 1998).

Recently, the presence of ApoE mRNA was demonstrated in some but not all neurons in the frontal cortex and in the hippocampus of normal controls and AD patients (Xu et al. 1999a, Zarow & Victoroff 1998). These findings suggested that neurons are able to synthesise ApoE and that the reported intracellular effects of ApoE may not only be mediated by neuronal uptake but also by intraneuronal synthesis (Ignatius et al. 1986, Nathan et al. 1994, Xu et al. 1999a).



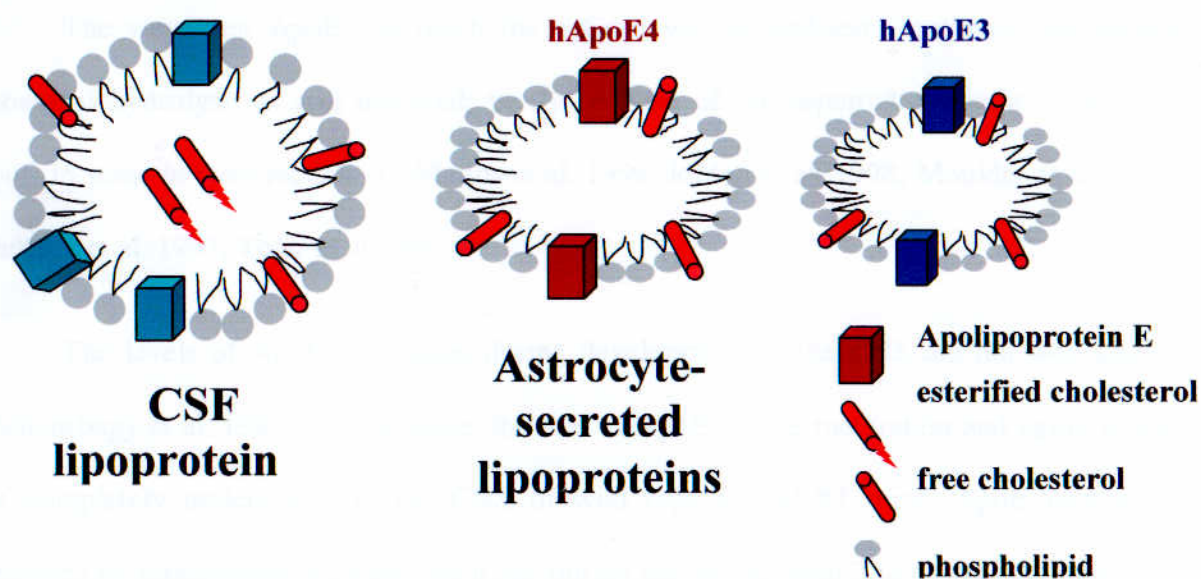


Figure 1.3 Characteristics of ApoE-containing lipoproteins in the brain. CSF lipoproteins are the size and density of plasma HDL. Nascent astrocyte particles from human transgenic mice are smaller and discoidal, with no esterified cholesterol in their core. Astrocytes from ApoE KO mice produce no detectable lipoprotein particles (Fagan et al. 1999, LaDu et al. 1998).

Receptors	Cell types	DG normal brain	DG AD brain	A $\beta$ plaque
<b>LDLr</b>	N, Ast	Neuropil <i>light</i>	Neuropil <i>light</i>	<i>rare</i>
<b>LRP</b>	N, Ast, $\mu$ glia choroid plexus	Neuronal <i>strong</i> (soma, axons, dendrites)	Neuronal <i>strong</i> Ast <i>strong</i>	<i>strong</i>
<b>VLDLr</b>	N, $\mu$ glia	Dendrites <i>moderate</i>	Dendrites <i>stronger inner 3<sup>rd</sup></i>	<i>Strong</i> in associate d $\mu$ glia
<b>Megalin</b>	Ependymal cells	NA	NA	NA
<b>ApoER2</b>	N	NA	NA	NA

Table 1 ApoE receptors and their expression in brain cells, particularly in the DG. N, neuron; Ast, astrocytes;  $\mu$ glia; microglia. Intensity of immunostaining is indicated in *italics*. NA, non available. Little is known of ApoE binding affinity with brain receptors. ApoE2  $\beta$ -VLDL enriched lipoproteins bind very poorly to the LDL receptor compared to ApoE3 and ApoE4, but they retain 40% of normal binding to LRP receptor (Kowal et al. 1990).

The view that ApoE can reach the cytosol via the endocytic pathway and escape lysosomal hydrolysis is still disputed; partly because of the reported cytosolic toxicity of ApoE in some *in vitro* models (DeMattos et al. 1999, Jordan et al. 1998, Moulder et al. 1999, Marques et al. 1997, Tolar et al. 1997).

The levels of ApoE expression during development of the CNS are not well known (Elshourbagy et al. 1985). Furthermore, the role of ApoE during maturation and aging is also not completely understood. In the CNS of wild type C57BL/6J mice, ApoE expression measured by immunoblot increases with age during the period from 2 to 16 months post-natal (Masliah et al. 1996). Both in rodents and humans, the localisation and level of expression of ApoE changes following brain injury or in pathological conditions as described in the following sections.

### **1.1.3 ApoE and neuronal injury.**

Clinical studies first established a strong association between the presence of the  $\epsilon 4$  allele and the effect of various forms of brain injury (reviewed in (Laskowitz et al. 1998)), such as intracerebral haemorrhage (Alberts et al. 1995), head injury (Teasdale et al. 1997), or chronic traumatic brain injury in boxers (Jordan et al. 1997). In all those cases, individuals with the  $\epsilon 4$  allele show a poorer prognosis for survival than those without an  $\epsilon 4$  allele. Following cardiopulmonary bypass surgery, patients with an  $\epsilon 4$  allele also suffer from greater post-operative cognitive deficits than those without an  $\epsilon 4$  allele (Newman et al. 1995). However, the mechanisms by which ApoE polymorphism may influence differentially the response and outcome to brain injury are still unclear and under investigation. Recently, some animal models were used to define the role of ApoE in response to brain damage. After global and transient ischemia injury in rodents, intraneuronal ApoE immunoreactivity is detected 6 to 24 hours respectively after the initial insult (Horsburgh & Nicoll 1996a, Horsburgh & Nicoll



1996b, Horsburgh et al. 1997, Kida et al. 1995). It is increased initially in astrocytes and neuropil, and later in ischaemic neurons (mainly CA1 pyramidal neurons in the hippocampus) and processes that are degenerating, probably via a neuronal uptake mechanism. Similar experiments performed on ApoE deficient mice produce increased neuronal damage compared to injured wild type controls, in various regions of the brain including the hippocampus (Chen et al. 1997, Horsburgh et al. 1999b, Laskowitz et al. 1997, Sheng et al. 1999), and suggest a protective role for ApoE. After focal ischemia, transgenic mice expressing the ApoE4 isoform have significantly larger cortical and subcortical infarct volumes compared with mice expressing the ApoE3 isoform (Sheng et al. 1998). In contrast, Horsburgh et al (Horsburgh et al. 1999a) found no influence of ApoE genotype on hippocampal neuronal damage, or on ApoE neuronal and glial immunoreactivity following global ischemia in human individuals. However, the inability to control for the severity and length of the ischemic episode, as well as survival time after ischemia in human cases, may account for this discrepancy. Although these experiments provide evidence that ApoE has a protective role after brain injury, its mechanisms of action are still speculative and may be plural. One favoured hypothesis seems to be its protective role against oxidative stress and subsequent lipid peroxidation occurring after global ischemia (Miyata & Smith 1996) as suggested by Horsburgh (Horsburgh et al. 1999b), and Sheng (Sheng et al. 1998). This possibility is supported by the fact that plasma lipoproteins from ApoE KO mice are more susceptible to *in vitro* oxidation than those of wild type mice (Hayek et al. 1994). Moreover, the diminished recovery in ApoE KO mice after closed head injury is related to their inability to counteract oxidative damage (Lomnitski et al. 1997). However, there is an alternative hypothesis for ApoE's protective role. ApoE is involved in membrane remodelling as a lipid carrier after neuronal injury (Poirier et al. 1991, Poirier et al. 1993a), and it also exerts a neurotrophic effect on neurons *in vitro* and *in vivo*, in an isoform-specific fashion (Bellosta et al. 1995, Buttini et al. 1999, Holtzman et al. 1995,

Nathan et al. 1994). Thus, ApoE could protect against neuronal injury by exerting its effects on neuronal survival and/or be involved in structural neuronal repair.

#### **1.1.4 ApoE and entorhinal cortex lesion.**

The entorhinal cortex (EC) contributes to the major part of neural inputs to the hippocampus. This large supply of multimodal information is sent into the hippocampus via the perforant path, as described in detail in section 1.2.1. Denervation of the dentate gyrus, due to loss of the perforant pathway projection from the EC, is proposed to contribute to the pathophysiology of AD (Geddes et al. 1985, Hyman et al. 1984, Hyman et al. 1986). Entorhinal cortex lesion (ECL) is another model of injury used in rodents, to examine the mechanisms associated with deafferentation and reinnervation. It is a classical example of reactive synaptogenesis, illustrated by the compensatory response of the hippocampus. ECL was shown to cause the loss of nearly 86% of the synaptic input to the granule cells of the dentate gyrus (Matthews et al. 1976). A few days after denervation, new synapses are formed, virtually replacing the lost inputs within a few weeks (Masliah et al. 1991a, Matthews et al. 1976). These new synapses originate from cholinergic septal neurons, glutamatergic commissural-associational hilar cells, and to a lesser extent from neurons of the contralateral EC (reviewed in (Deller & Frotscher 1997)). The early phase of reactive synaptogenesis and terminal proliferation is accompanied by an induction of ApoE gene expression in astrocytes of the deafferented zone, a concomitant decrease in activity of the HMG-CoA (3,3'-hydroxymethylglutaryl coenzyme A) enzyme involved in cholesterol synthesis and an increased expression of the LDL receptor in granule cells (Poirier et al. 1991, Poirier et al. 1993a). Ultrastructural studies show that throughout the 2-11 days post-lesion, astrocytes progressively engulf degenerating presynaptic terminals (Lee et al. 1977). Poirier (Poirier et al. 1993a) suggested that non-esterified cholesterol released during terminal breakdown is esterified, transported via the ApoE transport system to neurons undergoing reinnervation, and

taken up through the LDL receptor pathway where it would presumably be used as a precursor molecule for the synthesis of new terminals. Abnormal nerve regeneration following ECL has been reported in some ApoE deficient mice (Fagan et al. 1998, Poirier et al. 1993a, Stone et al. 1998, Teter et al. 1999a) but not in others (Anderson et al. 1998). In the latter case, other lipoproteins such as ApoJ may compensate for the loss of ApoE, as suggested by Popko's results (Popko et al. 1993) after sciatic nerve crush.

### **1.1.5 ApoE and structural plasticity in the nervous system.**

The first evidence that ApoE could influence neuronal structure was provided by Handelsmann et al (Handelsmann et al. 1992), showing that ApoE -rich  $\beta$ -VLDL lipoprotein (used as transport vehicle capable of delivering ApoE to receptor) and cholesterol, increase neurite outgrowth and branching of dorsal ganglion root neurons in culture. Further studies using the same *in vitro* system showed that neurons incubated with human ApoE3 and  $\beta$ -VLDL develop long neurites. In contrast, ApoE4 combined with  $\beta$ -VLDL results in the inhibition of neurite outgrowth (Nathan et al. 1994). Lipid-free ApoE has no effect on neurite outgrowth. Similar results are obtained on Neuro-2a cells (which are from a CNS derived neuronal cell line), when incubated with exogenous ApoE (Nathan et al. 1995), or when transfected to produce endogenous ApoE (Bellosta et al. 1995), both in presence of  $\beta$ -VLDL. To induce isoform specific effects on neurite outgrowth in these studies, ApoE has to associate with a transport lipoprotein vehicle directing it to the heparan sulfate proteoglycan (HSPG)-LDL-receptor related protein (LRP) complex. Before internalisation by cells, ApoE enriched lipoproteins bind first to cell-surface HSPG and then to the LRP (Bellosta et al. 1995, Holtzman et al. 1995, Mahley 1988, Mahley et al. 1994, Sun et al. 1998). However, Neuro2a cell lines expressing higher levels of ApoE isoforms produce a minimally lipidated ApoE3 isoform capable to stimulate neurite outgrowth in the absence of exogenous lipid (DeMattos et

al. 1998). In contrast, ApoE4 has no effect, and exogenous  $\beta$ -VLDL antagonises the stimulatory effect of ApoE3. These results suggest that in this case, neurite outgrowth may not be mediated by lipid delivery and may involve other ApoE receptors than the LRP.

Since  $\beta$ -VLDL lipoproteins are not found in the CNS, further studies were carried out with ApoE containing HDL-like lipoprotein from CSF or astrocyte-secreted, which are closer to the form of ApoE normally found in the brain (LaDu et al. 1998, Pitas et al. 1987). The results are similar to those obtained with cell lines (Fagan et al. 1996, Sun et al. 1998). Thus, when primary hippocampal neurons are grown in presence of astrocyte monolayers derived from human ApoE transgenic mice generated with the GFAP promoter, neurite outgrowth is greater from neurons grown in the presence of ApoE3-secreting astrocytes, compared with apoE4 secreting or ApoE knock-out astrocytes (Sun et al. 1998). As yet, the cascade of cellular events underlying the ApoE isoform-specific effects on neurite outgrowth is still unclear. However, ApoE3 stimulates the polymerisation of  $\beta$ -tubulin and stabilises the formation of microtubules in Neuro-2a cells, whereas ApoE4 apparently destabilises microtubule assembly (Nathan et al. 1995, Pitas 1996). Consistent with these findings, ApoE3 binds to microtubules and their associated proteins Tau and MAP2c *in vitro* whereas ApoE4 does less (Huang et al. 1994, Nathan et al. 1995, Strittmatter et al. 1994). However, such interactions have not yet been demonstrated *in vivo* and it is unclear how ApoE affects microtubule formation. It is well established that lipoprotein internalisation via the LDL receptor and the LRP is followed by an endocytic pathway that leads to lysosomal degradation (Krieger & Herz 1994). Although ApoE-enriched lipoproteins are also degraded by neurons, a portion of ApoE can apparently escape degradation as indicated by ApoE accumulation observed in cultured cells (Bellosta et al. 1995, Ji et al. 1998, Nathan et al. 1995). ApoE enriched lipoproteins can be internalised by 3 cellular mechanisms: the LDL receptor, the HSPG-LRP pathway, and a newly discovered HSPG-dependent/LRP-independent pathway (Ji

et al. 1998, Mahley 1988, Mahley et al. 1994, Weisgraber & Mahley 1996). Ji showed that this latter pathway is responsible for the differential retention of ApoE isoforms in the cells, and suggested that the ApoE4 isoform does not accumulate because it is recycled and released at the cell surface (Ji et al. 1998). These results led several authors to the following hypothesis (Ji et al. 1998, Lovestone et al. 1996, Mahley & Huang 1999, Nathan et al. 1995, Strittmatter et al. 1994, Weisgraber & Mahley 1996): ApoE3 may be internalised via the HSPG-dependent/LRP-independent pathway, escape degradation in the cytosol and complex with various cytosolic proteins to promote neurite outgrowth and microtubule stability. In contrast, ApoE4 may enter a pathway that favours release from the cells, resulting in decreased retention and ability to modulate neurite outgrowth or remodelling. To understand how the ApoE isoforms can exert their differential effects, it will be essential to characterise the intracellular localisation of ApoE in detail.

*In vivo*, dendritic integrity, assessed by MAP2 immunoreactivity, is preserved in the frontoparietal cortex and in the hippocampus of 12-month-old mice expressing the ApoE3 isoform, compared to wild type controls. In contrast, there is extensive disruption of the neuronal dendritic structure in ApoE deficient mice and to a lesser extent in ApoE4 mice (Buttini et al. 1999, Masliah et al. 1995, Veinbergs et al. 1999 but see Anderson et al. 1998 for no alteration in ApoE deficient mice). Although these results require confirmation, they support the view that ApoE3 maintains the neuronal integrity during aging better than ApoE4.

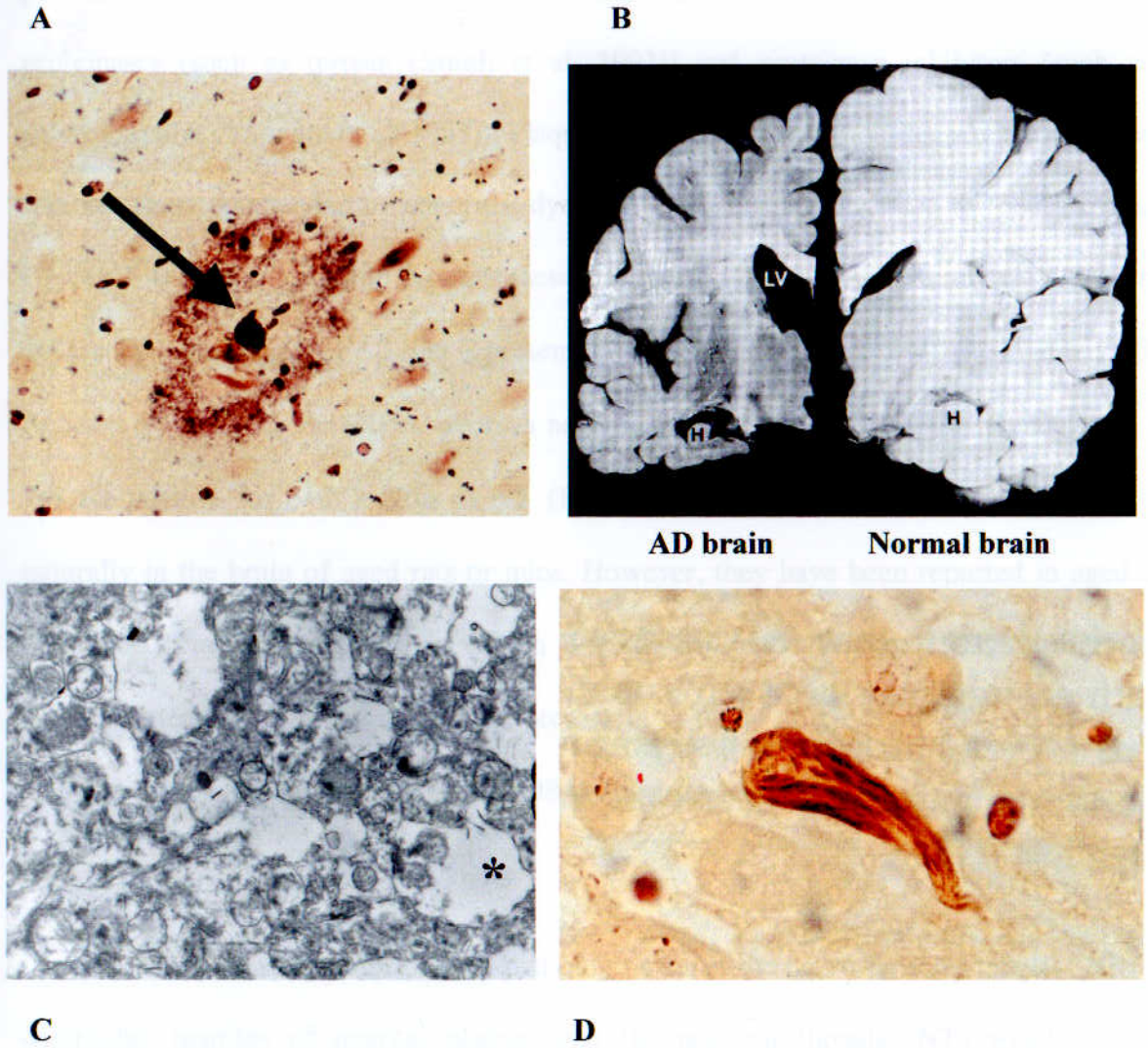
## **1.1.6 ApoE and Alzheimer's disease**

### **1.1.6.1 Alzheimer's disease**

Alzheimer's disease (AD) is the most common cause of dementia. In industrialised countries, its prevalence doubles every 5 years after age 65 with figures in the order of 4% of population over age 75, up to 32% over age 90 (Evans et al. 1989). This neurodegenerative

disease is characterised by a progressive cognitive decline and a loss of memory. The capacity to recall not only recent events, but also to learn and retain new information is severely affected. Reasoning ability and spatial and visuo-perceptual ability are also impaired, accompanied by language deficits and changes in behaviour (reviewed in (Grabowski & Damasio 1997)). The unequivocal diagnosis of AD relies however on histopathological evidence at brain autopsy or biopsy. Although the histological hallmarks of AD are well described, establishing criteria for the morphological diagnosis of AD is difficult because of the phenotypical heterogeneity of the disease, the absence of specific markers, and the overlap of AD pathology with changes observed in non-demented elderly individuals. Among the wide variety of morphological changes described in the brain of AD patients, amyloid plaques and neurofibrillary pathology are those used for the neuropathological diagnosis of AD, according to criteria based on age-related semi quantitative assessment (reviewed in (Jellinger 1998)).

A $\beta$  senile plaques and neurofibrillary tangles (NFT) are considered as the pathological hallmarks of the disease, despite the fact that they are not specific to it (reviewed (Adams & Duchon 1992, Esiri & Morris 1997)). Indeed, A $\beta$  deposits without NFT occur early in life in Down 's syndrome and other conditions, while in a variety of other disorders and normal aging, NFT are present with only few or no A $\beta$  deposits (Mann 1988, Simic et al. 1998, reviewed in (Jellinger 1998)).



*Figure 1.4 The pathological hallmarks of AD. Light microscopy pictures of a mature amyloid plaque with dense core (arrow) (A) and a neurofibrillary tangle in a pyramidal cell (D), both stained by the Bielschowsky method. B illustrates the atrophy of the brain: the lateral ventricle (LV) is enlarged whereas the hippocampus (H) is shrunken. Synaptic degenerative changes in neocortex neuropil (C) include distended axonal terminals (asterisk). Adapted from Gauthier et al, 1997, Allen and Dawbarn, 1995.*

### *Senile plaques*

Senile plaques mainly contain fibrillar aggregates of  $\beta$ -amyloid protein derived from the amyloid precursor protein (APP) (Esch et al. 1990, Sisodia et al. 1990), but also other proteins such as ApoE (Han et al. 1994b, Namba et al. 1991, Uchihara et al. 1995), a variety of proteinases (such as trypsin (Smith et al. 1993)) and proteinase inhibitors (such as  $\alpha$ 2-macroglobulin (Vangool et al. 1993)). Plaques without abnormal neurites are 'diffuse plaques' whereas those surrounded by abnormal dystrophic neurites are designed as 'neuritic plaques' (Figure 1.4). The relationship between these 2 types of plaques is not entirely clear. One major difference is the length of  $A\beta$  that is present. Diffuse plaques are  $A\beta$ 1–42 positive but  $A\beta$ 1–40 negative by antibody detection, whereas neuritic plaques may represent a more mature form and are positive for both lengths of  $A\beta$  (Iwatsubo et al. 1994). Senile plaques do not occur naturally in the brain of aged rats or mice. However, they have been reported in aged dogs, bears and primates (reviewed in (Finch & Sapolsky 1999, Walker 1991)). Infiltration of cerebral artery walls by  $\beta$ - amyloid is present in virtually all cases of AD and described as amyloid angiopathy (Glenner & Wong 1984).

### *Neurofibrillary pathology*

The 3 forms of neurofibrillary pathology include the neurofibrillary tangles (NFT), the dystrophic neurites of neuritic plaques and the neuropil threads (NT) which are found scattered in the neuropil (reviewed in (Esiri & Morris 1997, Terry et al. 1994)). All represent accumulations of insoluble paired helical filaments (PHF) which are composed of abnormally hyperphosphorylated microtubule associated Tau protein (Goedert 1993). In pyramidal neurons, NFTs accumulate in the cytoplasm in a flame shape and displace the nucleus (Figure 1.4). The PHFs are so insoluble that they remain within the neuropil as ghost tangles after the death and degeneration of the cell in which they developed. They become coated with other molecules such as GFAP, ubiquitin and ApoE (Yamaguchi et al. 1994).



### *Other lesions*

Hirano bodies and granulovacuolar degeneration are 2 other features of AD pathology that have been described but not well understood (see review by (Anderton 1997, Esiri & Morris 1997, Terry et al. 1994)). To complete the pathological picture of AD, there is a striking loss of neurons and synapses associated with cortical atrophy.

### *Neuronal loss*

Whereas in normal human aging only neurons from the dentate hilus and from the subiculum die, numbers of CA1 hippocampal cells and neurons from layers II and IV of the entorhinal cortex (EC) are severely diminished in AD (Davies et al. 1992, Gomez Isla et al. 1996, Hyman et al. 1984, Morrison & Hof 1997, West et al. 1994b). At the subcortical level, the cholinergic basal nucleus of Meynert and diagonal band of Broca also lose neurons, in parallel with reduced concentrations of acetylcholine (ACh) and acetylcholinesterase (AChE) activity in their projections zones, the hippocampus and the cortex (Davies & Maloney 1976, Perry et al. 1977, Whitehouse et al. 1982). The synthesis of ACh is assured by reaction of the choline acetyl transferase (ChAT) with choline. However, concentrations of choline and the activity of ChAT are greatly diminished in the brain of AD patients (Nitsch et al. 1992, Whitehouse et al. 1982). Moreover, cholesterol, which is required for the proper functioning of nicotinic receptor subtype is also reduced in the brain AD patients (Jones et al. 1988). The AChE inhibitor Tacrine was designed to take advantage of the residual cholinergic activity and provided temporary cognitive improvement in some AD patients but not all (Poirier et al. 1995, Poirier & Sevigny 1998).

### *Synaptic loss*

One major hallmark of AD is the massive loss of synapses. The molecular layer of the DG of the hippocampal formation is one of the regions most affected, and at a very early stage

of the disease. Reported synaptic decreases vary from 20 to 65% compared to aged-matched controls, depending on methods of assessment (electron or light microscopy) and severity of disease (Cabalka et al. 1992, Heinonen et al. 1995b, Masliah et al. 1994b, Sze et al. 1997). Other hippocampal fields and neocortical regions are also affected, but later in the disease process. Regions include the frontal cortex and temporal cortices, the nucleus basalis of Meynert and the locus coeruleus (Hamos et al. 1989, Honer et al. 1992a, Lassman et al. 1992, Lippa et al. 1992, Masliah et al. 1991c).

Several pathological changes occur at the synaptic site. At the ultrastructural level, most presynaptic terminals contain abnormally enlarged clear and dense-core vesicles. Others are dilated and contain increased numbers of normal-size vesicles, with occasional electron-dense bodies. In contrast, other presynaptic terminals show moderate to severe loss of presynaptic vesicles (Gonatas et al. 1967, Masliah et al. 1991b). Most presynaptic proteins such as Synaptophysin, Synapsin I and Rab3 for example, are severely and concomitantly depleted (Hamos et al. 1989, Heinonen et al. 1995b, Honer et al. 1992a, Lassman et al. 1992, Masliah et al. 1991b, Masliah et al. 1994b, Sze et al. 1997). Studies assessing synaptic loss by stereological counting have also established that synaptic loss is accompanied by an increase in mean synaptic size. This phenomenon has been observed in the frontal and temporal cortices as well as in the inner and outer molecular layer of the DG (DeKosky & Scheff 1990, Scheff & Price 1993, Scheff et al. 1996, Scheff & Price 1998), and is interpreted as a compensatory response to maintain a certain level of synaptic area, and therefore of synaptic transmission.

### *Atrophy*

Concomitantly with synaptic and neuronal loss, various regions of the brain are affected by atrophy during AD. Both white and grey matters are involved (Mann 1991). The

volumes of the hippocampus, the amygdala and the temporal lobe are significantly altered (De Toledo Morrell et al. 1997, Geroldi et al. 1999, Jack et al. 1992, Lehtovirta et al. 1995, Mann 1991, Yasuda et al. 1998). A detailed review is provided in section 3.2.

### *Neuropathological correlates of dementia and hypothetical aetiology of AD*

Although the picture of AD pathology is now very detailed, the aetiology of the disease is still unclear and several questions remain unanswered. What triggers the formation of senile plaques and of NFT? What is the mechanism of neuron death and synaptic loss? Are there relevant cause/effect relationships between all those features? Which lesions are responsible for memory and cognitive deficits? And ultimately, can we reverse the disease process? All these questions have been under intense scrutiny for the last decade but only partial answers have been obtained. In order to slow or prevent the disease, it is essential to identify the steps where pathogenesis could be inhibited.

The sequence of neurofibrillary pathology is predictable and shows little variation between AD patients. It can be divided into 6 stages, beginning with invasion of the transentorhinal cortex to finally affect other portions of the cerebral cortex and subcortical nuclei. The Braak staging system is based on location of damaged neurons and on the severity of changes (Braak & Braak 1991, Braak & Braak 1998). In general, initial NFT stages I and II develop in the transentorhinal cortex in the absence of A $\beta$  deposits and of clinical symptoms. The limbic stages III and IV show progression of neuritic lesions from the parahippocampal region to the hippocampus with preservation of the neocortex. The terminal stages V and VI represent fully developed AD with widespread devastation of the neocortex. Primary motor and sensory areas are the last to be affected. At that stage, patients are severely demented.

The question of whether plaques or tangles appear first, remains a matter of intense debate. According to Braak (Braak & Braak 1991), NFTs and NTs generally precede the formation of  $\beta$ -amyloid deposits in some components of the hippocampal formation (prosubiculum and CA1) and in the entorhinal region, whereas in others (DG and subiculum)  $\beta$ -amyloid usually appears before the formation of the neurofibrillary changes. Neocortical areas show accumulation of  $\beta$ -amyloid before the first appearance of NFTs and NTs. Size and shape of the deposits and their distribution pattern vary from one individual to another and are unrelated to age (Braak & Braak 1991, Fewster et al. 1991, Morris 1997). However, all investigators do not share this view. Some found that at very early stages of AD, patients have a pattern of tangle formation identical to that of non-demented aging. Although they occur in slightly greater densities in AD, individual variation and overlap exist, thus the differences may not be diagnostic (Morris et al. 1991, Morris et al. 1996b, Morris 1997, Price et al. 1991, Price 1993). In contrast, truly non-demented aging is associated with few, if any neuritic or diffuse plaques, whereas even in the earliest clinically detectable stages of AD, neocortical plaques are already prominent. This suggests that the presence of neocortical plaques, and particularly those with neuritic changes, best represent the disease process of AD. The possibility that a few NFTs or plaques in non-demented elderly could represent a preclinical stage of the disease rather than normal aging is still unresolved. Additional confusion is provided by the results of correlation studies. Several authors have correlated the severity of dementia with various neuropathological measurements such as plaque number, amyloid load, NFT number, synapse loss or hippocampal volume, and have inferred that one particular feature is more relevant to the pathology than the others (Arriagada et al. 1992, Bobinski et al. 1995a, Bobinski et al. 1995b, Cummings et al. 1996, DeKosky & Scheff 1990, Dickson et al. 1995, Hyman et al. 1986, Nagy et al. 1995b, Nagy et al. 1996, Samuel et al. 1994, Sze et al. 1997, Terry et al. 1991). Synaptic loss in various cortical regions and in the hippocampus is

generally accepted as the best correlate of dementia severity. However, this observation says little about disease pathogenesis. There are indications that the presence of NFTs is associated with synaptic loss (Callahan & Coleman 1995, Honer et al. 1992a, Lassman et al. 1992) while synaptic loss is no greater within diffuse plaques than in the neuropil outside them (Heinonen et al. 1995b, Masliah et al. 1990, Masliah et al. 1991b). However, it is severely accentuated within mature plaques (Masliah et al. 1990). Because synaptic loss is an early event in AD (particularly in the molecular layer of the DG) (Heinonen et al. 1995b, Hyman et al. 1986, Masliah et al. 1994b), Masliah suggested that the pathogenic process of AD begins with synaptic loss and neurodegeneration rather than with deposition of A $\beta$  protein (Martin et al. 1994, Masliah et al. 1991b, Masliah & Terry 1993, Masliah et al. 1993, Masliah et al. 1994a, Masliah 1995, Masliah 1998). In support of this theory, APP is normally present in synapses where it may play a role in synaptic stabilisation and neuroprotection, whereas in AD, APP abnormally accumulates in the plaque dystrophic neurites and in synaptic terminals (Masliah 1998). Neurodegeneration might follow via several hypothetical mechanisms, including 1) direct toxicity of one of its degradation products, A $\beta$ , 2) disruption of synaptic function or 3) deficient glutamate transporter activity resulting in glutamate neurotoxicity. Although there is evidence supporting this new theory, it is not generally accepted.

The discrepancies between results in the quest for the best correlate of dementia could arise through a number of possibilities: the variety of morphometric techniques used, how they are applied, which regions of the brain are studied, and the stage at which the disease is examined. Controversy may also originate from the phenotypic and genetic heterogeneity of the disease. Since the early 1990s, it has been shown that some forms of AD are associated with the inheritance of mutant genes, including those which encode the amyloid precursor protein (APP), and presenilin 1 and 2 (PS1 and PS2), or with the presence of specific alleles of apolipoprotein E (ApoE) (Chartierharlin et al. 1991, Corder et al. 1993, Goate et al. 1991,

Levy et al. 1995, Roses et al. 1994, Sherrington et al. 1995). Mutant APP, PS1 and PS2 are linked to relatively early cases of autosomal dominant familial AD (FAD) and are considered as causative genes whereas ApoE acts as a susceptibility gene, with the  $\epsilon 4$  allele increasing the risk of AD in a dose-dependent fashion in cases of late-onset AD (reviewed in (Esiri & Morris 1997, Wasco & Tanzi 1995)). Although AD is genetically heterogeneous, there is little or no difference in either the symptoms of dementia or in the pathology of the lesions in the brains of patients affected with the various types of AD (Lippa et al. 1996, Wasco & Tanzi 1995). Whilst this suggests that a common pathological mechanism may be involved, there is no reason to suspect that all the forms of AD, inherited or not, have necessarily identical pathogenic routes to similar neuropathology.

Despite the low proportion of familial AD caused by gene mutations, their biological significance is important because they all result in an increased production of A $\beta$ 1-42, which is the main component of amyloid deposition (reviewed in (Hardy 1997, Neve & Robakis 1998, Wasco & Tanzi 1995)). This finding, added to the fact that Down's syndrome patients develop the pathology of AD, and possess an additional chromosome 21 where the APP gene is located, pointed to a role of A $\beta$  in AD pathology. This gave rise to the amyloid cascade hypothesis as a possible pathological mechanism (Selkoe 1997). Although it is inappropriate here to develop fully the arguments in favour or against this theory (reviewed in (Hardy 1997, Neve & Robakis 1998, Wasco & Tanzi 1995)), it is important to describe briefly its main concept, since other factors such as ApoE may be involved, as explained in the following section. Selkoe (Selkoe 1997) summarised the theory as follows: "all gene defects lead to enhanced production, increased aggregation, or perhaps decreased clearance of A $\beta$  peptides. A $\beta$ 1-42, which is highly self-aggregating, would then accumulate, followed by A $\beta$ 1-40. The gradual cerebral build-up of A $\beta$  appears to result in local microglial and astrocytic activation,

with concomitant release of cytokines and acute phase proteins. These inflammatory changes or the direct toxicity of A $\beta$  would injure local neurons and their processes, causing profound metabolic changes. These could include altered Tau phosphorylation and PHF formation in some plaque-associated neurites and in neurons containing NFTs. Neuronal and glial injury would result in synaptic and neuronal loss, accompanied by several neurotransmitter deficits.”

Although there is little doubt that A $\beta$  plays a major role in the pathology of AD, several authors have challenged the idea that it is the primary cause of AD neurodegeneration. These issues have been reviewed in detail elsewhere by several authors (Esiri & Morris 1997, Hardy 1997, Neve & Robakis 1998). One major criticism is the lack of consensus regarding the mechanism by which A $\beta$  destroys neurons (Neve & Robakis 1998), and the observation that all but one transgenic models of A $\beta$  deposition failed to detect neuronal loss (Calhoun et al. 1998b, Duff 1998, Price et al. 1998). In addition, brains of some non-demented individuals also contains diffuse A $\beta$  deposits, and the total number of A $\beta$  deposits shows only a modest correlation with dementia (Arriagada et al. 1992, Gomez Isla et al. 1997, Nagy et al. 1995b). How A $\beta$  deposition could lead to neurofibrillary pathology is also unclear.

The present section is by no means a comprehensive review of AD pathology and lines of research. Several detailed books and reviews are available on the subject (Anderton 1997, Esiri & Morris 1997, Terry et al. 1994). This brief introduction of AD is however necessary to ‘set the stage’ for the following section dealing with the role of ApoE as a risk factor for AD. Indeed, although gene mutations provide an invaluable basis for research, they are responsible for only a very low proportion of AD cases. For example, mutations of the APP gene account for approximately 7% of early onset AD cases, which in turn represent only 15% of all AD cases (Wasco & Tanzi 1995). In contrast, the most common genetic predisposition for late AD is conferred by inheritance of the  $\epsilon$ 4 allele of the apolipoprotein E gene. The following section

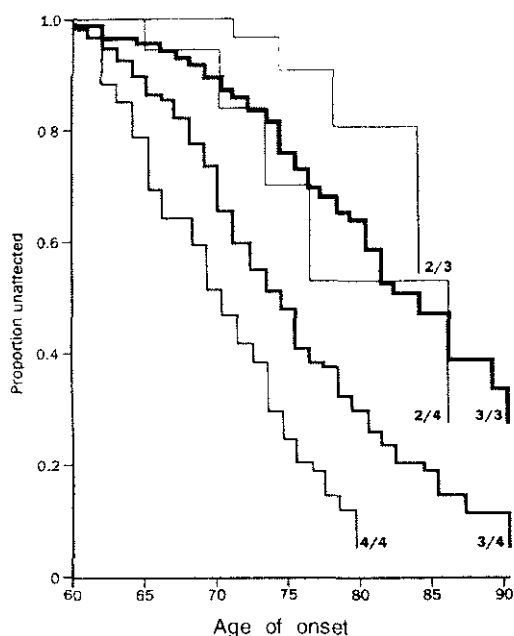
examines the evidence in favour of a role of ApoE in AD pathogenesis, and how it may feature in the current hypothetical mechanisms of the pathology.

#### **1.1.6.2 Possession of $\epsilon 4$ is a risk factor of AD**

In 1993, Allen Roses and his colleagues at Duke University demonstrated a 3-fold excess of the  $\epsilon 4$  allele for AD patients aged 65+ with (Corder et al. 1993) and without (Saunders et al. 1993b) close relatives also affected with AD. They showed that the risk of developing sporadic and late-onset familial Alzheimer's disease increases, and the age of onset decreases with the number of inherited  $\epsilon 4$  alleles (Figure 1.5). This association has since been confirmed worldwide, although the  $\epsilon 4$  allele frequency varies in different ethnic populations. Further investigation demonstrated a protective effect of the  $\epsilon 2$  allele for familial and sporadic AD (Corder et al. 1994, Lippa et al. 1997, Talbot et al. 1994, West et al. 1994a).

Survival from the onset of the symptoms of AD and the rate of progression of the disease are not influenced by ApoE polymorphism suggesting that the preclinical  $\epsilon 4$ -related processes do not continue during the clinical disease and do not cause more rapid decline among  $\epsilon 4$  carriers (Corder et al. 1993, Corder et al. 1994, Corder et al. 1995a, Hyman et al. 1996b but see Frisoni et al. 1995, Ohm et al. 1999, Stern et al. 1997).





*Figure 1.5 Risk of remaining unaffected by AD (late onset) in relation to ApoE genotype. From Corder et al 1998.*

However, the mechanisms by which ApoE isoforms are involved in the pathogenesis of AD are not yet elucidated. The finding that ApoE is detected in the characteristic lesions associated with this pathology, i.e. in extracellular senile plaques, intraneuronal fibrillary tangles and  $\beta$ -amyloid containing blood vessels (Namba et al. 1991, Rebeck et al. 1993, Schmechel et al. 1993, Strittmatter et al. 1993a, Wisniewski & Frangione 1992) opened new perspectives about the involvement of ApoE in AD, and an increasing number of studies now focus on the interactions of ApoE isoforms with key molecules of AD pathology. Thus, examining possible correlations between possession of the  $\epsilon 4$  allele and the number, size or degree of severity of the key lesions of AD has been a common approach. In fact, with the emergence of the amyloid cascade hypothesis, interest has been focused largely on association between the  $\epsilon 4$  allele and  $A\beta$  deposition.

### *ApoE and A $\beta$ deposition*

Several investigators demonstrated that brains from  $\epsilon 4$  AD patients contain significantly more amyloid plaques compared to  $\epsilon 3$  patients (Beffert et al. 1999, Hyman et al. 1995, Ishii et al. 1997, McNamara et al. 1998, Mann et al. 1997, Nagy et al. 1995a, Ohm et al. 1995, Oyama et al. 1995, Rebeck et al. 1993, Schmechel et al. 1993) with a clear gene dose effect, so that plaque density correlates positively with the number of  $\epsilon 4$  alleles. In contrast, other groups failed to demonstrate such a correlation, some suggesting that differences in ethnic populations, stages of pathology and technical considerations may be responsible for these discrepancies (Berg et al. 1998, Landen et al. 1996, Pirttila et al. 1997). *In vitro* studies have revealed multiple and complex relationships between A $\beta$  and the different ApoE isoforms. ApoE3 protects cultured cells from A $\beta$ -induced neurotoxicity, whereas ApoE4 does less (Miyata & Smith 1996), does not (Jordan et al. 1998) or even increases neurotoxicity (Ma et al. 1996). ApoE4 is more efficient than ApoE3 in promoting amyloid fibril formation (Castano et al. 1995, Ma et al. 1994, Strittmatter et al. 1993b). ApoE2 and E3 promote the binding and uptake of A $\beta$ 1–40 into chinese hamster ovary cells better than ApoE4 (Yang et al. 1999), while in hippocampal neurons, ApoE4 increases A $\beta$ 1–40 uptake (Beffert et al. 1998a) more than ApoE3. Conversely, A $\beta$ 1–40, A $\beta$ 1–42 and A $\beta$ 25–35 increase the binding of ApoE4-liposomes more than that of ApoE3 (Beffert et al. 1998a). *In vivo*, APP overexpresser mice crossed with ApoE deficient mice (APP+/-, ApoE-/-) form A $\beta$  deposits much later than APP overexpresser mice with normal ApoE levels (Bales et al. 1997), suggesting that ApoE influences A $\beta$  deposition *in vivo*. The same APP+/-, ApoE-/- mice crossed with ApoE3 or ApoE4 transgenic mice (whose astrocytes express physiological concentrations of human ApoE in the absence of mouse ApoE) dramatically suppress early A $\beta$  deposition *in vivo*, in contrast to the effect of mouse ApoE, or even when compared to mice lacking ApoE

(Holtzman et al. 1999). It is unclear why in this model, human and mouse ApoE produce opposite effects on A $\beta$  deposition. The authors suggested that each type of ApoE may have contrasting properties regarding A $\beta$  fibrillogenesis, or that human ApoE may promote A $\beta$  clearance better than mouse ApoE.

### *ApoE and NFT*

Studies on the association of the  $\epsilon$ 4 allele with NFT, the other major lesion of AD, have produced contradictory data. Initial reports indicated that the number of NFTs is greater in individuals with the  $\epsilon$ 4 allele than in those without (Beffert & Poirier 1996, Nagy et al. 1995b, Ohm et al. 1995) but such a correlation was not found by others (Berr et al. 1994, Gomez Isla et al. 1997, Landen et al. 1996, Landen et al. 1996, Morris et al. 1996a, Mukaetova-Ladinska et al. 1997, Oyama et al. 1995). In young individuals with initial neurofibrillary changes (Braak's stage I), the frequency of the  $\epsilon$ 4 allele is higher than in aged matched controls devoid of such changes (Ghebremedhin et al. 1998). Consistently, in normal individuals and AD patients, the  $\epsilon$ 4 allele is associated with a higher stage of neurofibrillary formation compared to aged-matched  $\epsilon$ 3 patients (Ohm et al. 1999). This suggests that the  $\epsilon$ 4 allele not only influences the age of onset of the disease, but also possibly contributes to the formation of initial neurofibrillary changes at a young age. The molecular mechanisms behind the association of the  $\epsilon$ 4 allele and the formation of NFT are still unknown. *In vitro*, ApoE3 interacts with Tau whereas ApoE4 does not, suggesting that ApoE3 may prevent Tau phosphorylation, and allow it to function normally in microtubules. In the presence of ApoE4, Tau would not be protected from hyper-phosphorylation, becoming inactive and forming NFTs (Lovestone et al. 1996, Strittmatter et al. 1994). However, it is debatable whether ApoE can interact with Tau or tubulin from within the cytosol because 1) ApoE is a lipid-binding protein and it is unclear how it could exist in a lipid-free form in the cytoplasm, and 2) ApoE

would have to escape lysosomal degradation after internalisation if it was exogenous to the neuron.

### *ApoE expression in AD*

The cellular localisation of ApoE is indeed crucial to understand its involvement in the pathology of AD. It is still unclear whether the degree of neuronal ApoE transcription and expression differs between normal non-demented controls and AD patients (Diedrich et al. 1991, Oyama et al. 1995, Yamada et al. 1995, Zarow & Victoroff 1998). ApoE expression level in  $\epsilon 4$  AD patients is either reduced (Bertrand et al. 1995, Yamada et al. 1995, Beffert, 1999) or not different from that of non- $\epsilon 4$  carriers (Han et al. 1994a, Hesse et al. 1999). ApoE is present not only in NFTs, but also in the vulnerable neurons that do not bear NFTs, in the brain of AD, Parkinson's disease patients and in non-demented aged controls (Han et al. 1994b). A recent study showed that in 3 visual cortical areas (Einstein et al. 1998), the distribution of intraneuronal ApoE paralleled the regional distribution of pathological changes that occur during AD. The presence of intraneuronal ApoE at the earliest Braak and Braak stages (Braak & Braak 1991) in non-demented control brains suggested that it could represent a normal characteristic of neocortical neurons, or be a sign of neuronal challenge or injury, marking preclinical AD. The fact that the cerebellum (which is a relatively spared structure in AD compared with cerebral cortex) displays a very strong ApoE transcription in glial cells but none in neurons, indicates that ApoE gene expression in neurons may be one factor or marker for selective vulnerability of cerebral cortical neurons observed in AD. The significance of ApoE accumulation in injured neurons remains to be determined. It may represent an initial protective response to injury by providing cholesterol and lipids for neuronal repair.

In addition to its presence in different categories of cells, ApoE is also found in the cerebrospinal fluid (CSF) associated with lipoproteins and it is produced within the blood-

brain-barrier (Linton et al. 1991, Pitas et al. 1987). As yet, there is no agreement about the levels of CSF ApoE in AD. Some authors have reported no change (Lefranc et al. 1996) or a decrease (Blennow et al. 1994, Lehtimäki et al. 1995) associated with AD, suggesting that this measurement may not be useful for the diagnosis of Alzheimer pathology.

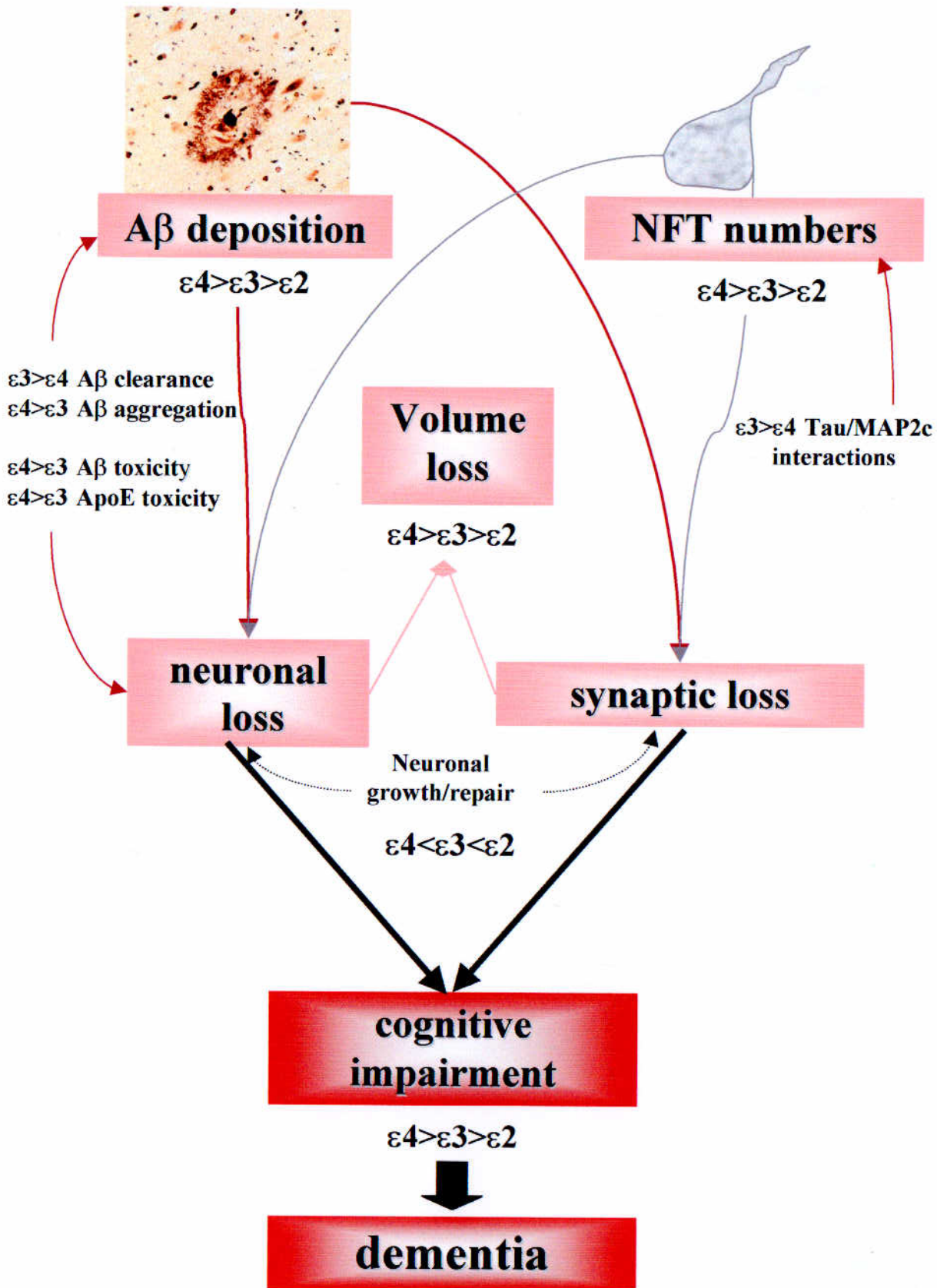
#### *ApoE, cholinergic dysfunction and treatment of AD*

The cholinergic system is severely affected by AD. Moreover, the maintenance of this system relies heavily on lipid delivery to synthesise ACh (Nitsch et al. 1992). This suggested that the role of ApoE in lipid transport could be central to the pathology in  $\epsilon 4$  AD patients. ApoE associates with lipoproteins transporting essential lipids for the ACh system (LaDu et al. 1998, Fagan et al. 1999). Amongst them are phosphatidylcholine (PC) and phosphatidylethanolamine (PE). These brain membrane phospholipids, are both donors of choline, which itself is a rate-limiting precursor of ACh. Cholesterol, which is required for the proper functioning of nicotinic receptor subtypes (Jones et al. 1988), is also transported via ApoE- enriched lipoproteins (Mahley 1988). It is interesting that  $\epsilon 4$  AD patients have reduced levels of PC and PE, as well as increased levels of products from their breakdown in the brain (Klunk 1998). In addition,  $\epsilon 4$  AD patients show a reduced activity of enzyme responsible for the synthesis of ACh, ChAT, reduced nicotinic receptor binding sites compared to non- $\epsilon 4$  carriers (Arendt et al. 1997, Poirier et al. 1995 but see Svensson et al. 1997), and reduced number of cholinergic neurons in the nucleus basalis of Meynert (Arendt et al. 1997, Poirier et al. 1995 but see Salehi et al. 1998). This general degradation of the cholinergic system may be due to impaired lipid delivery in  $\epsilon 4$  carriers, resulting in either degradation of membrane after use of their PC to synthesise ACh, or in the contrary, shut-down of ACh synthesis and cholinergic neurotransmission to preserve membrane integrity and plasticity (Beffert et al. 1998b, Poirier 1994, Poirier et al. 1995). ApoE deficient mice have decreased PC levels and

display abnormal cholesterol distribution in brain membranes preparations (Igbavboa et al. 1997, Lomnitski et al. 1999). In addition, most ApoE deficient mice failed to detect any significant alteration, as assessed by ChAT activity, ACh release, nicotinic and muscarinic receptor binding sites and AchE staining (Anderson & Higgins 1997, Fagan et al. 1998, Krzywkowski et al. 1999, Parker et al. 1996, but see Chapman & Michaelson 1998, Gordon et al. 1996a). Taken together, these findings favour the hypothesis that a compensatory mechanism may alter membrane lipid composition to maintain the integrity of the cholinergic system.

According to the hypothesis that  $\epsilon 4$  AD patients have an impaired cholinergic system, cholinergic drug therapies, such as Tacrine, which are based on the functional use of residual cholinergic functions, are expected to be more efficient in non- $\epsilon 4$  carriers. However, such an effect is still unclear (Poirier et al. 1995, Poirier & Sevigny 1998, Mac Gowan et al. 1998).

Taken together, these findings suggest a number of ways in which ApoE polymorphism could influence the severity of AD pathology. Figure 1.6 illustrates ApoE interactions with the various components of this neuropathology.



## **1.2 ApoE and cognitive impairment.**

This section first examines how morphological parameters could account for memory and cognitive deficits observed during aging and the pathology of AD, and then reviews the current findings linking ApoE polymorphism to cognitive impairment.

### **1.2.1 Morphological correlates of cognitive impairment.**

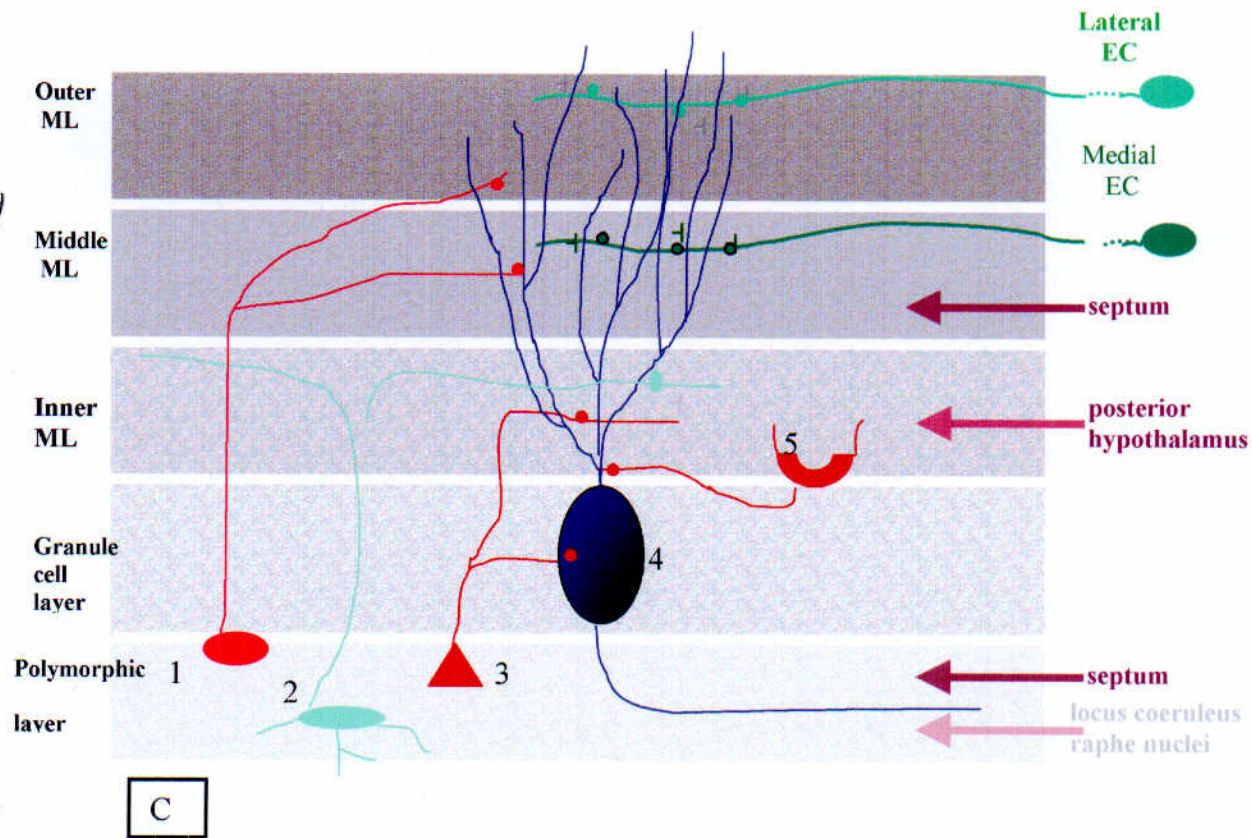
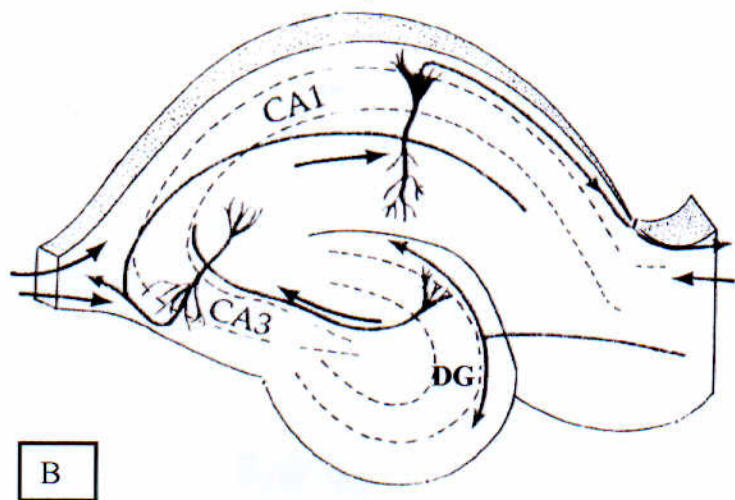
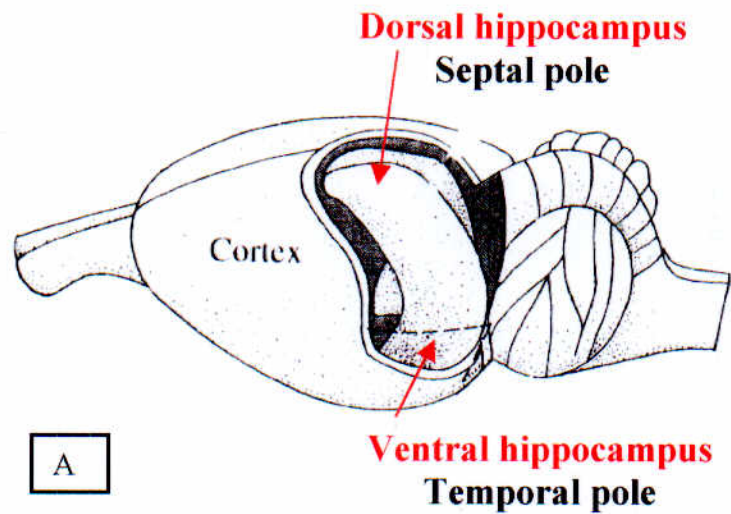
AD, and often aging too, are accompanied by deficits in learning and memory in humans and animals. Attempts to define the neurobiological substrates of memory dysfunction have led to the precise description of morphological changes that occur in the brain during AD and aging. An important feature of memory impairment in both cases is the selective vulnerability of new information. In contrast, the capacity to recall information acquired in the distant past is preserved (Rempel Clower et al. 1996, Teng & Squire 1999). This deficit is similar to that attributed to bilateral hippocampal damage in man.

Attention to the mnemonic functions of the temporal lobe was raised by the human case HM. After bilateral surgical removal of the medial temporal lobe, HM suffered from severe anterograde amnesia (Scoville & Milner 1957). Since then, several cases of human amnesia have confirmed and extended the findings with monkeys and rodents. They show that bilateral damage limited to the CA1 region of the hippocampus is sufficient to produce moderately severe anterograde memory impairment, and that bilateral damage to the hippocampal formation beyond the CA1 region can produce even more severe anterograde as well as retrograde amnesia covering up to 15 years (Squire & Zola 1996). In rodents, electrophysiological recordings, and selective lesions of the hippocampus disrupting the acquisition of spatial tasks, indicate that the dorsal hippocampus but not its ventral part plays a special role in spatial learning (Bontempi et al. 1999, Hampson et al. 1999, Moser & Moser 1998, Wood et al. 1999). In parallel with lesion studies, functional imaging in normal human



brain have confirmed the importance of the hippocampus for other forms of memory, by demonstrating hippocampal activation during the encoding of verbal and visual information (Fernandez et al. 1999, Rempel Clower et al. 1996, Squire & Zola 1996, Teng & Squire 1999).

In AD, the neuroanatomical distribution of lesions makes it clear that the hippocampus is functionally disconnected from the cerebral cortex early in the disease (Hyman et al. 1984). Before expanding on this subject, it will be helpful to introduce briefly the anatomy of the hippocampus. Figure 1.7 describes the intrinsic and extrinsic connectivity of the hippocampus. The entorhinal cortex is the only cortical region to project to the hippocampus. The cell bodies of its layers II and III send their axons to the outer two thirds of the molecular layer of the DG, forming the perforant path (PP) and making synapses with the dendritic branches of the granule cells of the DG. The DG projects in turn to the cells of the CA3 region, which connect pyramidal cells of the CA1 region. Outputs from CA1 neurons reach several subcortical and cortical regions, including the subiculum and the amygdala. Examination of temporal lobe structures from AD patients show that cells from layers II and IV of the EC as well as CA1 and subicular neurons are affected by NFT and senile plaques early in AD pathology (Braak & Braak 1991, Hyman et al. 1986). By disrupting cortico-hippocampal and hippocampo-cortical connections, AD pathology functionally isolates the hippocampus from the cortex, leading to the early signs of memory impairment. Consistently, the molecular layer of the DG is one of the regions most affected by synaptic loss, probably as the result of neuronal death of the EC cells (Cabalka et al. 1992, Heinonen et al. 1995b, Masliah et al. 1994b, Sze et al. 1997). Again, this event occurs very early in the disease and has been strongly correlated with memory impairment (DeKosky & Scheff 1990, Samuel et al. 1994, Sze et al. 1997, Terry et al. 1991). Whilst the rationale for early memory impairment in AD seems apparent in humans, this is not the case for age-related cognitive impairment in non-human animals which do not develop AD nor its lesions.



*Figure 1.7 The anatomy of the hippocampus (after Amaral & Witter 1989, Amaral 1995, Teyler & Discenna 1984).*

**A.** The hippocampal formation comprises 4 cortical regions. These include the dentate gyrus (DG), the hippocampus proper (which can be divided into 3 subfields, namely CA3, CA2 and CA1), the subicular complex (which can also be divided into 3 subdivisions: the subiculum, presubiculum and parasubiculum) and the entorhinal cortex (EC) which, particularly in rodents, is generally divided into medial and lateral subdivisions. The 3 dimensional shape of the rodent hippocampal formation appears as an elongated structure with its long axis bending in a C-shape manner from the septal nuclei rostro-dorsally to the temporal lobe caudo-ventrally. The long axis is generally referred to as the septotemporal axis and the orthogonal axis is referred to as the transverse axis.

**B.** The hippocampus circuitry consists of a largely unidirectional transverse loop of excitatory pathways through the DG, CA3, CA1 and subiculum. The DG comprises 3 layers. Closest to the hippocampal fissure is a relatively cell free layer called the molecular layer (ML), underlined by the principal cell layer made up primarily of densely packed columnar stacks of granule cells (4). The granule cells and the molecular layer form a U- shape structure enclosing a cellular region called the hilus or polymorphic layer, which constitutes the 3<sup>rd</sup> layer of the DG. The dentate granule cell has an elliptical cell body with a characteristic cone-shaped tree of spiny dendrites whose branches are all directed towards the edge of the molecular layer. Neurogenesis of granule cells occurs throughout the life of rodents and humans but decreases with age (Kuhn et al. 1996). Along the deep surface of the granule cell layer are several other cell types with distinctively different somal shapes and dendritic configuration. The synaptic connections in the ML are mostly organised in a 3-zone layer. The inner 3<sup>rd</sup> of the ML receives a projection that originates exclusively from cells in the polymorphic layer, principally glutamatergic mossy cells (2), on the ipsi and contralateral sides. The majority of terminals form asymmetric, presumably excitatory synaptic contacts on spines of the granule cell dendrites. Some symmetric, presumably inhibitory, synaptic contacts on the granule cell bodies and apical shafts arise from different cell types localised in the DG, among which basket (3) and chandelier (5) cells. Within the outer 2 thirds of the ML, perforant path synapses make up at least 85% of the total synaptic population while remaining inputs originate from subcortical regions and local cells (GABA/somatostatin cells (1)). Neurons from layers II and III of the EC provide the major input to the DG via the perforant pathway, as well as terminating in all the other subdivisions of the hippocampus. After leaving the EC, the fibres enter the underlying white matter and the angular bundle. They then traverse the subiculum and cross the hippocampal fissure to enter the DG. The terminals of the PP fibres are strictly confined to the outer or superficial 2 thirds of the ML, where they form asymmetric synapses. These occur most frequently on the dendritic spines of the granule cells. Fibres originating from the lateral EC area terminate in the outer one third of the ML whereas fibres from the medial EC area terminate in the middle one third of the ML. This transverse system features glutamatergic excitatory en passage synapses where a single afferent fibre makes a series of synaptic contacts on the dendrites of numerous cells as the axons proceeds along its transverse trajectory rather than a cluster of terminations in a single location. Many astrocytes are scattered in the molecular layer (Kosaka & Hama 1986)

**C.** Although the intrinsic pattern of connectivity repeats itself all along the septotemporal axis of the hippocampus, afferents and efferents connectivity changes from the dorsal to the ventral pole. The lateral, intermediate and medial zones of the EC project topographically to distinct and non-overlapping regions along the longitudinal axis of the DG. Since the 3 zones receive specific sets of cortical and subcortical inputs, this segregation of hippocampal afferents provides only the dorsal hippocampus but not the ventral part with visual, auditory and somatosensory inputs. It was suggested that different kinds of information are processed at different longitudinal levels of the hippocampus.

To date, a wide variety of mammals including monkeys, polar bears and dogs, naturally develop A $\beta$  deposition with neurocytoskeletal abnormalities but no evidence of NFT (Finch & Sapolsky 1999, Walker 1991). However, laboratory rodents are the exception and do not develop such pathology. Several species show cognitive deficits at an old age. In the aged dog, cognitive dysfunction correlates with A $\beta$  accumulation (Cummings et al. 1996) whereas in monkeys, it correlates better with myelin breakdown and loss of white matter (reviewed in Peters et al. 1996). However, as in humans, there are individual differences, and some monkeys or rats show more impairment than some others at the same age. Those which show the least impairment can be regarded as aging ‘successfully’ (Rapp & Amaral 1992). Amongst the animal models developed to study the neurobiology of aging, rodents offer a relatively short life span, small size and high fertility. A number of behavioural tasks can test memory and cognitive performance. Spatial navigation tasks in various mazes are commonly used to detect decline in spatial memory, which is particularly vulnerable with advanced age and is sensitive to hippocampal damage (Ingram & Jucker 1999, Mac Donald & Overmier 1998, Squire & Zola 1996).

Because the neuron is the basic functional unit of the brain, several investigators have counted neuronal numbers in specific brain regions known to be involved in cognitive deficits associated with aging and AD, such as the hippocampus. A common hypothesis is that specific neuronal loss may be responsible for cognitive alterations. Such belief was supported by early human studies showing a negative correlation between age and neuronal density in the neocortex, indicating up to 40% neuronal loss during aging (Brody 1955). This view has since been seriously challenged because of technical issues. Haug (Haug et al. 1984) established that preparation of brain tissue for microscopy causes young cortical matter to shrink more than aged tissue, and therefore considerably biased measurements of neuronal density. Moreover, appropriate stereological methods for neuron quantitation have been unavailable until recently,

making earlier data questionable. Nowadays, changes in neuronal number are assessed with unbiased stereological tools. It is now clear that in aged humans, neuronal loss is limited to specific brain regions only. There is no substantial loss of the principal neurons of the hippocampus (Davies et al. 1992) and in the EC (Gomez Isla et al. 1996, Morrison & Hof 1997) but a decrease of neuron numbers in subiculum and the hilus of the DG (West 1993). This pattern is clearly different from that of AD, where neuronal loss severely affects the CA1 region of the hippocampus and layers II and IV of the EC (Hyman et al. 1984, Hyman et al. 1986, West et al. 1994b).

Recent studies have evaluated the relationship between the ability of aged rats to learn a hippocampus-dependent task such as the Morris water maze task, and the number of principal hippocampal neurons. For each of the hippocampal fields, neuron number was preserved in aged rats as a group and in aged individuals with spatial learning deficits, in comparison with young rats (Rapp & Gallagher 1996, Rasmussen et al. 1996). Thus a loss of principal neurons does not occur in the hippocampus of old rats and cannot account for age-related deficits in hippocampus-dependent learning and memory. In C57BL/6 mice, there is also no loss of neurons in CA1 or in DG and there is no age-related deficit for the Morris water maze task (Calhoun et al. 1998a).

Other candidates as a substrate for learning and memory deficits are changes in synapse number and in synaptic efficacy. There is a growing body of evidence that memory processes are mediated by structural changes in synapses as well as enhancement of synaptic strength (Bailey & Kandel 1993, Doubell & Stewart 1993, Hunter & Stewart 1993, Patel & Stewart 1988, Rusakov et al. 1993, Rusakov et al. 1995, Rusakov et al. 1997, Stewart & Rusakov 1995, Stewart et al. 1998). However, estimation of synaptic densities in several brain regions and in different species have produced inconclusive data (see Table 2). Again, only

recently developed stereological tools provide reliable data and studies using these techniques are still in a minority. Attempts to correlate cognitive status with synapse density are also rare. Cognitive and learning abilities in mice can be affected by aging and mouse strains (Jucker & Ingram 1997, Owen et al. 1997, Roullet & Lassalle 1995). C57BL/6 mice are commonly preferred for the production of transgenic mice. Both their performance in the water maze and the number of synaptophysin positive boutons in the ML of the DG or in CA1 are largely preserved during aging (Calhoun et al. 1998a, Ingram & Jucker 1999). Moreover, there is a positive correlation between water maze performance and number of DG synaptophysin-positive boutons. Rats and monkeys show age-related cognitive decline, however they only display minor or regionally specific synaptic loss. These observations first suggested that synaptic loss may not underlie cognitive decline. However, many technical issues need to be raised. Very few studies have used stereological methods to count synapses and few hippocampal sub-fields have been examined. Moreover, when synaptic proteins levels were measured, often the whole hippocampus was dissected. The absence of a correlation between protein levels and performance at specific learning and cognitive tasks may be due to the fact that functional specificities of the hippocampal sub-regions, as well as the functional distinction between dorsal and ventral hippocampus, were not taken into account. Ultrastructural studies provide the most definitive assessment of synaptic structural changes, however they do not detect alterations in the specific synaptic or receptor proteins which lead to changes in functional connections. For example, Barnes' physiological studies in aged rats have shown that a decrease in synaptic input (consistent with synaptic loss) to the DG from the EC is accompanied by an increase in the strength of existing connections (Barnes 1994, Barnes 1999). Finally, the relationship between synaptic strength and levels of synaptic proteins is unknown. Therefore, studies using levels of synaptic markers as an index of synaptic connectivity are difficult to interpret.

Investigators	Method for synapse quantification	Species	Brain regions investigated	Synapse (all kinds) changes		Correlation with cognitive deficits
				density	Size	
(Geinisman & Bondareff 1976)	EM	rat	MML of DG	↓	N/A	N/A
(Geinisman et al. 1977)	EM		IML of DG	↓	N/A	N/A
(Bertoni Freddari et al. 1986)	EM		IML of DG	↓	↑	N/A
(Geinisman et al. 1992)	EM + stereology		IML and MML of DG	↓	N/A	N/A
(DeGroot et al. 1995)	EM + stereology		CA3	↓	↑	N/A
(Saito et al. 1994)	ELISA/ Synaptophysin		Entorhinal cortex hippocampus and other regions			N/A
(Nicolle et al. 1999)	Immunoblot Synaptophysin/ Synaptotagmin/ SNAP25		Hippocampus	⇔		NO correlation
(Smith et al. 1999)	Counting Synaptophysin immunolabelled boutons		Hippocampus (all subfields) CA3, OML and MML of DG	⇔ ↓		NO correlation Positive cor. with deficit
(Calhoun et al. 1998a)	Counting Synaptophysin immunolabelled boutons	mouse	DG and CA1	⇔		No deficit but positive cor. between performance and boutons number

(Tigges et al. 1995)	EM	monkey	OML of DG	↔	↔	N/A
(Tigges et al. 1996)	EM + stereology		IML of DG	↔	↔	N/A
(Gibson 1983)	EM	human	Frontal cortex	↓	N/A	N/A In non dem. elderly
(Adams 1987)	EM		Precentral motor cortex	↓	↑	N/A In non dem. elderly
			Postcentral somatosensory cortex	↔	↔	N/A
(Bertoni Freddari et al. 1990)	EM		IML of DG	↓	↑	N/A In non dem. elderly
(Bertoni Freddari et al. 1990)	EM		Frontal cortex	↔	N/A	N/A In non dem. elderly
(Scheff & Price 1993)	EM		Temporal lobe	↔ but neg cor size/n		N/A In non dem. elderly
(Bertoni Freddari et al. 1996a)	EM		Cerebellum	↓	↑	N/A In non dem. elderly
(Scheff et al. 1996)	EM + stereology		OML of DG	↔ but neg cor size/n		N/A In non dem. elderly
(Scheff & Price 1998)	EM + stereology		IML of DG	↔ but NO cor size/n		N/A In non dem. elderly
(Hamos et al. 1989) (Lippa et al. 1992)	Densitometry/ ICC synapsinI/ synaptophysin		OML/IML of DG	↔		N/A In non dem. elderly
(Sze et al. 1997)	Imm.blot/ synaptophysin		CA1, Entorhinal cortex and others	↔		N/A In non dem. elderly

*Table 2 Synaptic changes associated with aging across species. EM, electron microscopy; DG, dentate gyrus; N/A, non available; co size/n., correlation between synaptic size and synaptic number; non dem., non demented; neg.,negative; OML, outer ML; IML, inner ML*



### 1.2.2 Influence of ApoE polymorphism on memory and cognitive impairment

Studies of learning, memory and other cognitive functions in non-demented elderly demonstrate that individuals with the  $\epsilon 4$  allele have poorer performance than non  $\epsilon 4$  carriers, whereas those with the  $\epsilon 2$  allele perform better than all the others genotypes. Follow-up examination of the same groups show that subjects with the  $\epsilon 2$  allele maintain their cognitive performance, whereas that of subjects with other genotypes deteriorates with age (Berr et al. 1996, Bondi et al. 1995, Feskens et al. 1994, Helkala et al. 1995, Helkala et al. 1996, Henderson et al. 1995, Hyman et al. 1996a, Kuller et al. 1998, Reed et al. 1994, Soininen & Riekkinen 1996, Yaffe et al. 1997). These data suggest that inheritance of the  $\epsilon 4$  allele is associated with an increased chance of developing cognitive impairment and dementia, whereas inheritance of the  $\epsilon 2$  allele is associated with a smaller risk for developing cognitive impairment. However, it is important to note that many  $\epsilon 4$  individuals reach old age without cognitive deficit. Increased numbers of NFTs, increased A $\beta$  deposition and hippocampal atrophy described in the brain of  $\epsilon 4$  patients (see section 1.1.6.2) may form decades before the onset of dementia, and may increase the risk of cognitive decline before the clinical diagnosis of dementia. Hyman also reported a stronger effect of the  $\epsilon 4$  allele on women's cognitive decline, compared to men (Hyman et al. 1996a). This finding is in agreement with recent studies in 2 different lines of human ApoE transgenic mice, where 6 month old hApoE4 females show some cognitive deficit in the water maze task and age-matched males are not impaired (Raber et al. 1998, Veinbergs et al. 1999). In contrast, most lines of ApoE knock-out mice display cognitive deficits, that may be related to alteration of the integrity of cholinergic system for some, and to neurodegeneration in the neocortex and the limbic system for others (Gordon et al. 1995, Gordon et al. 1996a, Krzywkowski et al. 1999, Masliah et al. 1997, Masliah et al. 1999, Oitzl et al. 1997, Raber et al. 1998, Veinbergs et al. 1999, Veinbergs & Masliah 1999 but see Anderson et al. 1998).

### 1.3 Use of transgenic mice to study the role of ApoE

In the present thesis, the approach chosen to investigate the role(s) played by ApoE in the neuronal and synaptic integrity of the hippocampus is the study of ApoE deficient mice, as well as transgenic mice expressing the different human isoforms. The rationale behind the use of knock-out mice is as follows: one knocks-out a gene *in vivo* and creates a mutant mouse that completely lacks the gene of interest. The comparison of the phenotypes between wild type (WT) and knock-out (KO) mice should then provide clues about the function of the protein of interest, assuming that no other protein/gene takes over the function of the missing gene. Several ApoE knock-out lines have been developed in the last few years. They have sometimes produced contrary results, leaving several issues unresolved, as discussed in previous paragraphs and reviewed in Veinbergs & Masliah (1999). Moreover, WT mice express a single ApoE isoform, which shares only 70% homology with the human ApoE4 amino acid sequence (Rajavashisth et al. 1985). Consequently, the influence of ApoE polymorphism cannot be examined directly. ApoE cellular localisation is another major difference between rodents and primates/humans: ApoE mRNA and expression are observed in neurons and astrocytes in primate and human brains whereas they are only astrocytic in rodent brains under normal conditions (Boyles et al. 1985, Diedrich et al. 1991, Han et al. 1994a, Han et al. 1994b, Ignatius et al. 1986, Metzger et al. 1996, Poirier et al. 1991, Rebeck et al. 1993, Xu et al. 1996, Xu et al. 1999a, Zarow & Victoroff 1998). These crucial differences have led to the development of 'human' transgenic mice to mimic better the physiological expression of ApoE in the human brain. To date, 5 lines of human transgenic mice have been created. They differ mainly in the promoter driving the ApoE gene, the number of copies of the transgene, the level of expression, and the organ and cellular localisation. Table 3 describes the characteristics of the different lines.

Mouse line	Background	Promoter	Isoforms-copy number	In Organ	Expression Cellular distribution In brain	Level In brain
(Xu et al. 1995) (Xu et al. 1996)	ApoE KO (From C57BL/6J)	Human ApoE	E2-205 8 copies E3-437 2 copies E4-81 2 copies	Serum, brain, liver, kidney, heart, muscle and others	Neuronal and glial (astrocytic)	Not quantified
(Bowman et al. 1995) (Bowman et al. 1996)	Wild type?	Human Transferrin	E2 3 copies E3 40 copies E4 2copies	Brain and liver	Glial (astrocytic)	E3 249 E2 78 E4 21 pmol/g of brain homogenate
(Sun et al. 1998)	ApoE KO (From C57BL/6J)	GFAP	E3-2 N/A E4-22 N/A	Brain	Glial (astrocytic)	Not detailed 0.12 to 0.3 µg/mg protein
(Smith et al. 1998)	ApoE KO (From FVB/N and C57/BL6)	Human GFAP	E2 N/A E3a-b N/A E4a-b-c N/A	Brain and plasma	Glial (astrocytic)	E2 1.12 E3 0.2 to 1.97 E4 0.72 to 1.54 µg/mg protein
(Buttini et al. 1999)	ApoE KO (From C57BL/6J)	Rat Neuron-specific-enolase (NSE)	E3 E4	Brain	Neuronal	E3 4.4 E4 4.6 µg/mg protein

*Table 3 Current models of ApoE transgenic mice*

The ApoE transgenic lines chosen here offer several advantages over other lines. They were constructed using large human DNA fragments containing the ApoE, ApoC1 and ApoC1' pseudogene, with human promoters and tissue expression elements in an ApoE knock-out mouse background (Xu et al. 1995). This way, only human ApoE is produced and there is no influence of endogenous mouse ApoE to confound the results of any experiment. Moreover, the pattern of ApoE expression is 'humanised' for each ApoE isoform transgenic line: ApoE mRNA and protein are detected in neurons as well as in glial cells (Roses et al. 1998, Xu et al. 1995, Xu et al. 1996). If the mouse ApoE gene is substituted with human ApoE gene by target replacement, the human gene is placed under normal mouse promoter and mouse expression elements, and results inadequately in a 'rodent' pattern of ApoE expression as observed in other lines (Roses et al. 1998, Xu et al. 1996). Because ApoE may be involved in cytoplasmic mechanisms in AD and neuronal repair after head injury, both in glial and neuronal cells, this characteristic pattern of expression in the transgenic line used in this study represents a major advantage over other constructs. In ApoE knock-out mice, which lack ApoE-mediated cholesterol transport, blood concentrations of cholesterol are 6 to 7 times higher than those found in wild type mice. Of all the human transgenic lines produced by Dr Xu, we chose those with ApoE isoforms where cholesterol concentrations were similar and substantially corrected. It appeared that these 3 lines were constructed with different copy numbers of the ApoE gene and consequently, differences in levels of expression can be clearly observed. Although this may represent a major limitation of this transgenic model, the unique 'humanised' pattern of ApoE expression of these lines seems an essential advantage to study possible impairment in the maintenance of neuronal and synaptic integrity, where neuronal intracellular mechanisms and interactions between ApoE and other proteins may be involved.

It is important to note that despite the obvious link between the  $\epsilon 4$  allele and AD, human ApoE transgenic mice are not, in any case, a model of this disease. Only A $\beta$  over-

expresser mice lead to A $\beta$  deposition similar to that observed in the disease (reviewed in (Duff 1998, Price et al. 1998)), whereas, for example, such events have not been reported in any existing ApoE transgenic mice (Smith et al. 1998). A $\beta$  over-expresser mice still represent incomplete models of AD because they do not develop typical NFT or marked neuronal loss (Duff 1998, Price et al. 1998). However, crossings between various transgenic mice to produce double transgenic animals seem promising. For example, A $\beta$  over-expresser crossed with human ApoE mice have recently been produced to examine the influence of ApoE polymorphism on A $\beta$  deposition in the brain (Bales et al. 1997, Holtzman et al. 1999). If human ApoE transgenic mice crossed with other transgenic mice are to be extensively studied in the future, it will be essential to characterise fully each line first. Hopefully, the results presented in this thesis will add to the basic knowledge necessary to pursue such complicated double transgenic studies.

## 1.4 Objectives

The previous sections demonstrated that ApoE is a multifunctional protein with important roles in neuronal plasticity and repair. ApoE polymorphism modulates the risk of developing AD and the age of onset of this disease, and it is also associated with various degrees of severity of pathology. Moreover, it influences the prognosis of recovery and the extent of neuronal damage and cognitive impairment after head injury. With the risk of neuronal damage increasing with age (in the context of AD or head trauma), it seems reasonable to think that the influence of ApoE polymorphism on neuronal maintenance is age-dependent.

Multiple interactions of ApoE with key molecules have been described. However, it is still unclear which of the multiple functions of ApoE is critical to result in these polymorphic effects. Poor regenerative capacity of the  $\epsilon 4$  allele after neuronal damage seems to be a solid hypothesis, although interaction with other molecules such as  $A\beta$  may also be relevant and are not exclusive.

*Transgenic mice* have become a very popular tool not only to study the function of specific proteins, but also as an attempt to model disease pathogenesis for the development of therapeutic drugs. It is clear that species differences prevent the direct comparison between mice and humans, leading to the production of 'humanised transgenic mice', i.e. mice carrying one or several human genes coding for proteins of interest. Human ApoE transgenic mice are useful not only to study ApoE polymorphism itself, but also for the future production of double or triple transgenic mice expressing other protein such as  $A\beta$ , towards better models of AD pathology. However, such studies first require a basic knowledge of single transgenic mice, to be able to interpret results in multiple transgenic models.

Since synaptic and neuronal integrity are important markers of neuropathology, the aim of this thesis was to determine the influence of ApoE polymorphism on structural synaptic morphometric parameters, such as synapse number and size, throughout the life of human ApoE transgenic mice. The DG was chosen as the region of interest because of its early and well-known vulnerability in AD, and its capacity for synaptic plasticity.

Towards this purpose, the following goals were established:

- 1) Compare qualitatively gross (brain and hippocampal volumes) and fine (ultrastructural) neuronal and synaptic morphology between the different groups of mice (WT, ApoE KO and human transgenic mice)
- 2) Determine the normal pattern of structural synaptic changes during aging in WT mice.
- 3) Make identical measurements in ApoE KO mice to infer a possible role of mouse ApoE in structural synaptic plasticity during aging.
- 4) Make identical measurements in human ApoE transgenic mice to examine the influence of human ApoE polymorphism in structural synaptic plasticity during aging.
- 5) Examine the influence of human ApoE polymorphism on synaptic and dendritic content of glutamate in the DG, to gain some information about the status of synaptic transmission efficacy associated with ApoE genotype.

All the measurements were performed with unbiased stereological methods at the electron microscope level to produce precise, reliable and comparable data.

## **Chapter 2   Materials and methods**



## 2.1 Transgenic mice

Identical lines of mice were obtained from colonies bred at Duke University Medical Centre (DUMC), North Carolina, USA and at Glaxo-Wellcome, Stevenage, UK.

Wild type (WT) mice were C57BL/6J. ApoE knock-out (ApoE KO) mice were produced by Dr Maeda (UNC, Chapel Hill, NC) (Piedrahita et al. 1992). Embryonic stem (ES) cells from 129J mice were isolated. Their normal mouse ApoE gene was then disrupted by removing a DNA fragment containing part of the ApoE gene's exon and intron 3, and replacing it by the neomycin-resistance gene in a targeting plasmid. After homologous recombination between the endogenous ApoE locus and the targeting plasmid, the modified ES cells were injected into host blastocysts from C57BL/6J mice and implanted into foster mothers. The F1 resulting animals inherited a normal and a modified ApoE allele and were backcrossed at least 6 times to C57BL/6J mice to remove the 129J background and increase the representation of the C57BL/6J background. This process creates a strain that carries the ApoE mutated gene on the desired wild type background (Gerlai 1996b).

Heterozygous human ApoE transgenic mice were generated by Dr. Xu (DUMC, Durham, NC, USA) as described in detail in Xu et al (Xu et al. 1996). Briefly, DNA fragments containing human regulatory sequences and the coding sequences for the 3 isoforms of human ApoE were isolated. They were microinjected independently into single-celled embryos fertilised by ApoE KO fathers, and from mothers that were a cross of strains C57BL/6J and DBA/2J. The resulting founder mice inherited a normal mouse ApoE allele from the injected female and an inactivated allele from the male ApoE KO donor, and also possessed the human ApoE transgene. They were bred to ApoE KO mice to produce animals hemizygous for human ApoE transgene and homozygous for KO mouse ApoE gene. Human ApoE hemizygous transgenic animals were bred to homozygosity and back-crossed further at least 6

times to ApoE KO mice to remove the genetic influence of DBA/2J background strains. The presence of human ApoE genes was confirmed by polymerase chain reaction. However, their localisation in the mouse genome has not yet been carried out. The mode of integration and copy number of transgenes were analysed by Southern blotting. Serum cholesterol concentrations in ApoE KO mice were 6 to 7 times higher (756 mg/dl) than that found in WT mice (98 mg/dl). In hApoE2, E3 and E4 mice, cholesterol concentrations were substantially corrected and very similar (168 to 173 mg/dl).

In the present study, 76 mice were used, including males as well as females. The detailed composition of the groups is given in Table 1. The following transgenic lines were used: ApoE2- line 205 (8 copies of the human ApoE transgene), ApoE3- line 437 (2 copies), ApoE4-line 81 (2 copies). Mice were separated into 3 age groups: young (from 6 to 10 months), adult (from 14 to 18 months) and aged (from 19 to 24 months).

Human Apolipoprotein E (hApoE) and adult ApoE KO mice were taken and perfused at DUMC in April 1997 and 1998, while young and aged ApoE KO and WT mice were obtained on a regular basis from July 1996 to January 1998 from colonies transferred from DUMC, NC, USA to Glaxo-Wellcome, Stevenage, UK. Data from mice from both sources were pooled for statistical analysis of the results on the assumption, that since the mice were bred in very similar conditions (low-fat diet, 12h dark/light cycle), the geographical localisation of the animal houses would not be a major factor of variability in the results.

The research using animals described in this thesis complied with the Home Office Animals (Scientific Procedures) Act 1986. The numbers of animals were the minimum possible to demonstrate reliable effects.

	Number of animals	Gender males/females	Age range (months)
<b>Wild type</b>	17	17 / 0	
<i>young</i>	6	6 / 0	8
<i>adult</i>	5	5 / 0	16
<i>old</i>	6	6 / 0	21
<b>ApoE KO</b>	16	15 / 1	
<i>young</i>	6	6 / 0	8
<i>adult</i>	4	4 / 0	17
<i>old</i>	6	5 / 1	22 to 24
<b>hApoE2</b>	13	7 / 6	
<i>young</i>	4	0 / 4	10
<i>adult</i>	3	1 / 2	14 to 16.5
<i>old</i>	6	6 / 0	19 to 22
<b>hApoE3</b>	7	3 / 4	
<i>young</i>	0	–	–
<i>adult</i>	7	3 / 4	14 to 18
<i>old</i>	0	–	–
<b>hApoE4</b>	23	17 / 6	
<i>young</i>	6	4 / 2	6
<i>adult</i>	11	7 / 4	15 to 17
<i>old</i>	6	6 / 0	19 to 20

*Table 4 Profiles of all mouse groups.*

## **2.2 Perfusion.**

Mice were anaesthetised with an intraperitoneal injection of sodium pentobarbitone (0.1ml/30g body weight of SAGATAL 60 mg/ml) and then perfused transcardially with 15 ml of 0.9% saline solution followed by 150 ml of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer at room temperature (See Appendix for solutions recipes). A peristaltic pump delivered the solutions at a flow rate of 4.1ml/min. The whole brains were removed from the skull and placed in fresh fixative overnight at 4°C.

Tissue sampling differed for subsets of animals, depending on which experiments they were included in. This is described in detail in section 2.3 for hippocampal and brain volume estimation and in section 2.4 for electron and light microscopy.

## 2.3 Volume estimation.

The volume of the brain and the volume of both right and left hippocampi were estimated for a subgroup of 15-19 month old mice (detailed in Table 5) using the Cavalieri method (Cavalieri 1635, Howard 1998). Each brain was sectioned horizontally using an Oxford vibrating microtome into 150  $\mu\text{m}$  sections. One in three sections was stained with a solution of 0.01% toluidene blue in 0.1M phosphate buffer for 15 min and then washed with buffer, resulting in 10- 12 sections from the whole brain (see Appendix for solutions recipes). The sections were left in 0.1M phosphate buffer at 4°C for 2 days so that the cell layers were very distinct. Each section was mounted on a glass slide, scanned with a Umax flat-bed scanner and printed on paper to a magnification of 10. The delineation of the hippocampus was made according to well-known architectonic and topographic data (Sidman et al. 1971) and included Ammon's horns, the DG and the subiculum. The olfactory bulbs and the cerebellum were excluded from the measurement of brain volume. The areas of the hippocampi and the brain were estimated by counting the numbers of points that hit a quadratic lattice superimposed on the image. The size of the grid in relation to the size of the printed image was such that approximately 100 and 200 points for the hippocampus and the brain respectively, were counted in the 10 to 12 sections. The calculated areas, the section thickness and the distance between sections were then entered in the Cavalieri formula to calculate the volume:  $V = T \times a/p \times \sum_i^m P_i$  where  $T$  is the distance interval between 2 sections,  $a/p$  is the area associated with each point of the grid and  $\sum_i^m P_i$  is the number of points landing within the transect of the region of interest (hippocampus or whole brain) on the  $i^{\text{th}}$  section (see Figure 2.1)

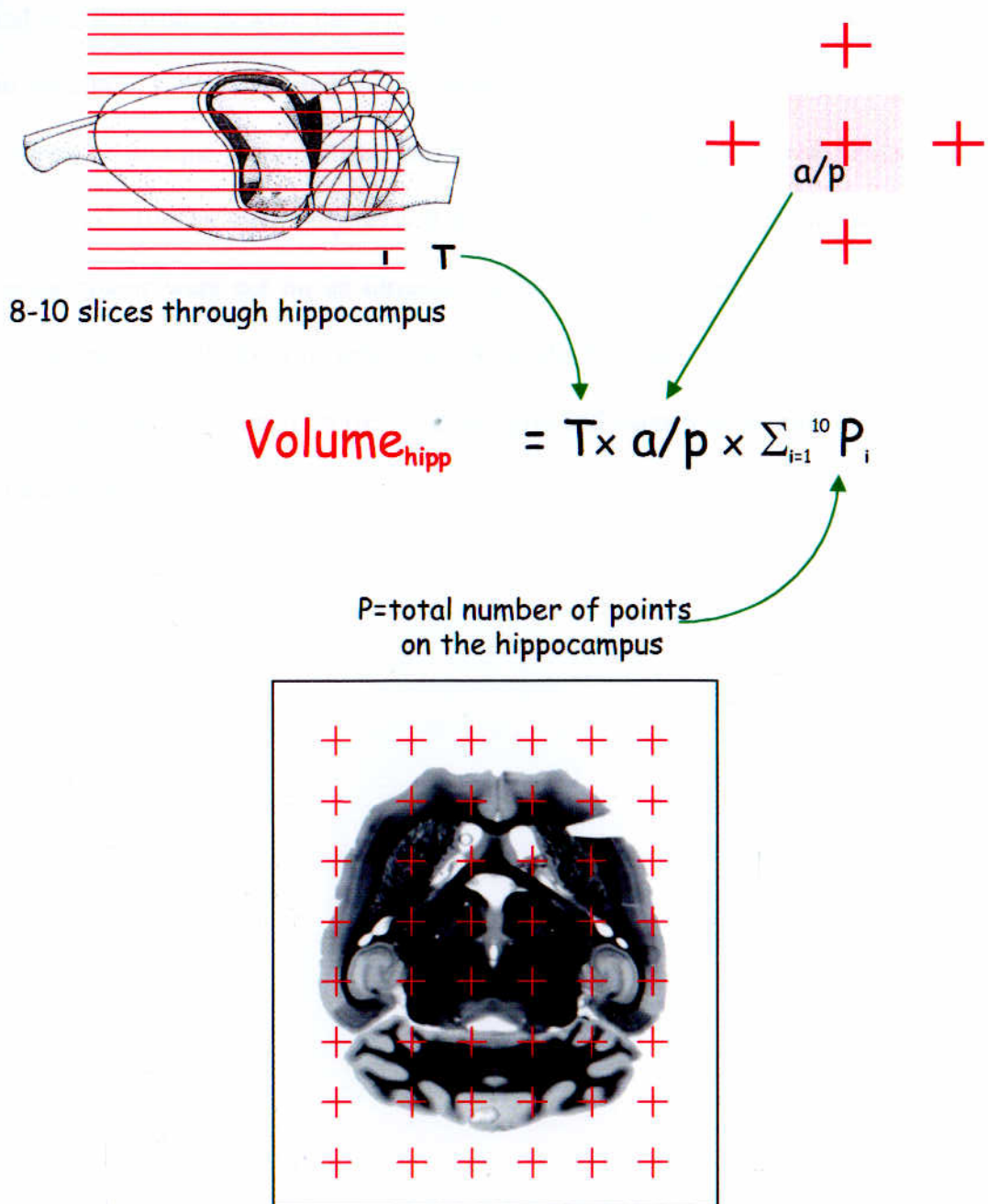


Figure 2.1 Schematic representation of the procedure used to calculate the hippocampal volume with Cavalieri's method (see text for explanation).

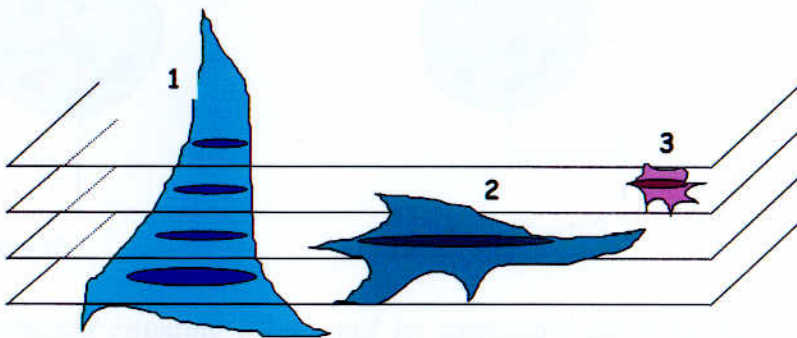
## **2.4 Electron microscope processing.**

3 transverse slabs of 1mm thickness across the entire dorsal hippocampus from both right and left hemispheres were dissected for each animal. The tissue was then prepared for electron microscopy by postfixing in 1% osmium tetroxide for 1 h, dehydrating through a graded series of acetone before embedding in Epon resin (see Appendix for solution recipes and procedures). Blocks were polymerised at 60°C for 24-48 h. Ultrathin sections of silver interference colour were cut on an ultramicrotome with a diamond knife and mounted on 2x1mm slot grids with Pioloform/carbon (see Appendix for solution recipe) support film. Pairs of serial sections were mounted on the same slot grid and stained with uranyl acetate and lead citrate (see Appendix for procedure).

## 2.5 Morphometry.

In all scientific work involving microscopy on mammals and particularly electron microscopy, the entire object of interest i.e. the hippocampus cannot be examined. It is therefore necessary to take a sample of the tissue to make an estimate of the number required (Howard 1998, Mayhew 1996, West 1999). In the present study, a block from the dorsal hippocampus was taken at random to estimate synapse density, neuron density and total apposition zone area per unit volume (Figure 2.5).

Earlier methods for estimating particle numbers relied on individual sections. However, arbitrary particles cannot be counted without bias from such sections, as these methods relied on assumption about particle shape, size and orientation (Figure 2.2). The only reliable way to obtain such estimates is by means of unbiased stereology (Howard 1998). Stereology is a technique that enables one to obtain data on the number of objects in a 3-dimensional structure by sampling in 2 dimensions.

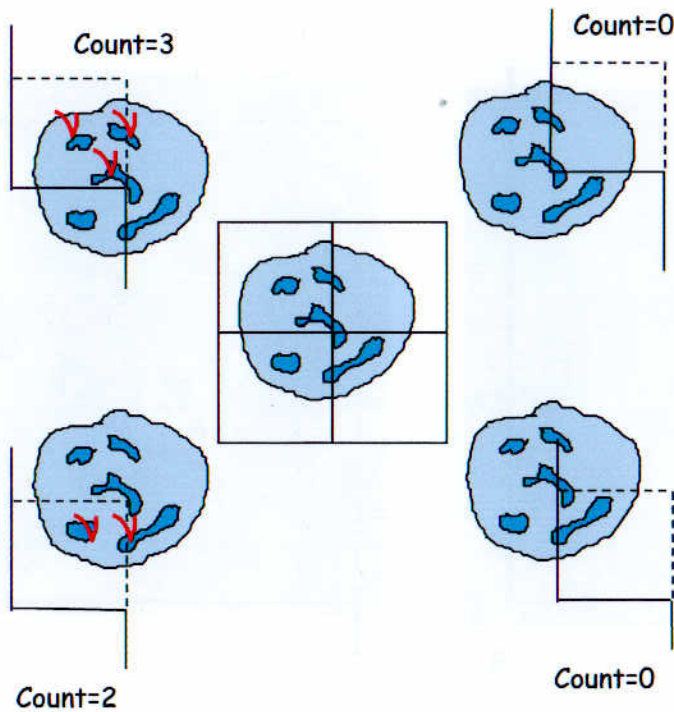


*Figure 2.2 Illustration of 3D neurons being intersected by a series of sectioning planes, represented by the set of lines. The number of times a neuron is intersected by the set of planes depends on its height normal to the plane of sectioning or its orientation. Although 1 and 2 have approximately the same dimensions, neuron 1 is cut 4 times whereas neuron 2 is only cut once as a consequence of its orientation. If the interval between the planes was doubled, the small neuron 3 would go uncounted. To avoid this source of bias, neurons are not counted in planes but in volumes by using the disector (adapted from (Howard 1998)).*



### 2.5.1 The disector

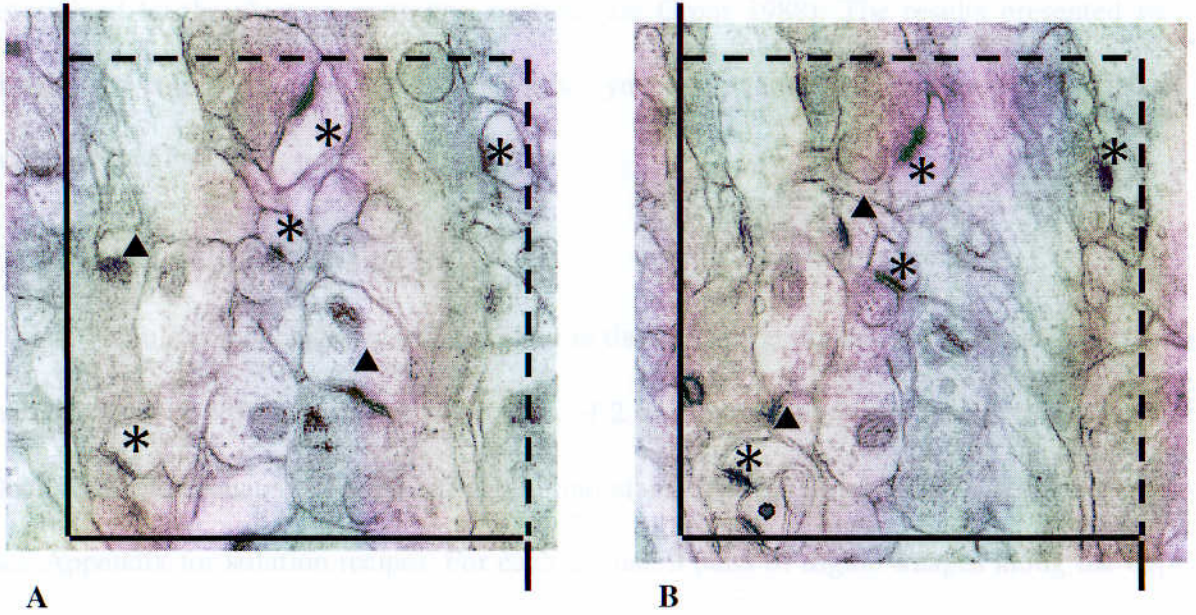
The disector (Sterio 1984) consists of a pair of registered serial sections a known distance apart. The method relies on the principle that if a particle transect (from a synapse or a neuron in the present case) is seen in one section and not the next, it is counted. The counting frame consists of a solid 'forbidden line' which extends above and below the field of view to infinity, and a dashed acceptance line. Any object that is cut anywhere by the forbidden line is not counted. Objects falling fully inside the counting frame or those that cut the acceptance line without also cutting the forbidden line are counted (Figure 2.3).



*Figure 2.3 The unbiased counting frame and its associated counting rule (adapted from (Howard 1998)). The application of this rule is such that if a bounded 2D object containing 2D particles was completely covered by the tessellation of counting frames, each 2D particle is counted only once.*

One of both serial sections is designed as the reference section and the other as the look-up section. For each transect correctly sampled by the counting frame within the reference section, a corresponding transect is sought in the look-up section. If none can be

found, the particle is counted in 3-D and is designed as 'specific'. The count is associated with a volume of space equal to the area of the counting frame multiplied by the distance between the sections (i.e. their thickness). The role of reference and look-up section can be reversed, therefore doubling the efficiency of the disector. The formula for estimation of numerical density is written as follows:  $N_v = \Sigma Q^- / (A.T) \cdot \Sigma P$  where  $\Sigma Q^-$  is the total number of counted profiles that appear only in the look-up section, T is the section thickness, A is the area of the counting frame and  $\Sigma P$  is the number of disectors (12 per mouse in the present study).



*Figure 2.4 Illustration of the use of the physical disector. If section A plays the role of reference section, synapses marked by an asterisk are present on both sections and therefore are not counted. Synapses marked by a triangle ( $n=2$ ) are only present on section B, the look-up section, and are therefore counted. The synapse marked by a circle is crossing the forbidden line and is not counted. The roles can be swapped so that section A is the look up section and section B the reference section, generating a new disector with 2 synapses counted.*

### **2.5.2 Mean synapse density $N_{v\ syn}$**

Synapse density per cubic micron was estimated using a disector in the MML of the DG (Figure 2.4 and Figure 2.5). Twelve pairs of digital images of the sections were acquired along the axis of the cell layer, from a JEM 1010 electron microscope at a magnification of x 12000 using a Kodak Megaplug digital camera. On the digital images, synapses were identified by the presence of both pre- and post-synaptic densities, with an associated vesicle cloud in the presynaptic bouton. They were then classified as shaft or spine synapses depending on the nature of their postsynaptic site. The sum of shaft and spine synapse densities provided the total synaptic density for each mouse. The section thickness was determined by the electron scattering method (De Groot 1988). The results presented were obtained by counting a total of 14482 specific synapses (average of 190 synapses per mouse) in the MML of the DG.

### **2.5.3 Mean neuron density $N_{v\ nrn}$**

Granule cell density per cubic micron in the DG was estimated using the same disector method. Pairs of adjacent transverse sections of 2  $\mu\text{m}$  thickness were prepared from the same blocks used for synapse density estimation and stained with 1% toluidene blue in 5% borax (see Appendix for solution recipe). For each animal, 3 pairs of digital images along the upper limb of the DG were acquired from a light microscope at a magnification of x 80 using a microscope mounted camera interfaced to a Joyce-Loebl Magiscan MD system. 5330 specific neurons in total (average of 70 neurons per mouse) were counted in the granule cell layer of the DG.

### **2.5.4 Mean number of synapses per neuron $Syn/nrn$**

Combining estimates of  $N_{v\ syn}$  and  $N_{v\ nrn}$  allows the unbiased estimation of the mean number of synapses per neuron,  $Syn/nrn = N_{v\ syn} / N_{v\ nrn}$ .

### 2.5.5 Total apposition zone (AZ) area $S_v$

The determination of the total apposition zone (AZ) area per cubic micron  $S_v$  was estimated using another unbiased stereological method (Rusakov et al. 1998, Underwood 1970). All the postsynaptic densities (PSD) profiles were observed on the same digital images used to estimate the synapse density. In each sampling frame, all identified PSD profiles were outlined as *curvilinear binary segments using cursor editing tools*. The background image was cut off using grey level thresholding. The accumulated length of all curvilinear segments was automatically measured in each image using an algorithm developed in the laboratory and stored on a file. This accumulated length was then divided by the frame area, to obtain the PSD length per unit section area,  $L_A^*$ . Finally, the value of total AZ area,  $S_v$ , was derived directly from  $L_A$  (Underwood 1970):  $S_v = 4.(L_A) / \Pi$  where  $L_A$  is the mean value of  $L_A^*$  across the sample of digital images. In total, 8 to 10 frames per mouse were analysed, to provide 90 to 110 profiles per animal.

### 2.5.6 Mean apposition area per synapse Mean AZ/syn

Combining estimates of  $S_v$  and  $N_{v \text{ syn}}$  allows the unbiased estimation of the mean apposition zone area of individual PSDs, in other word the area of individual synapses: Mean AZ/syn =  $S_v / N_{v \text{ syn}}$ .



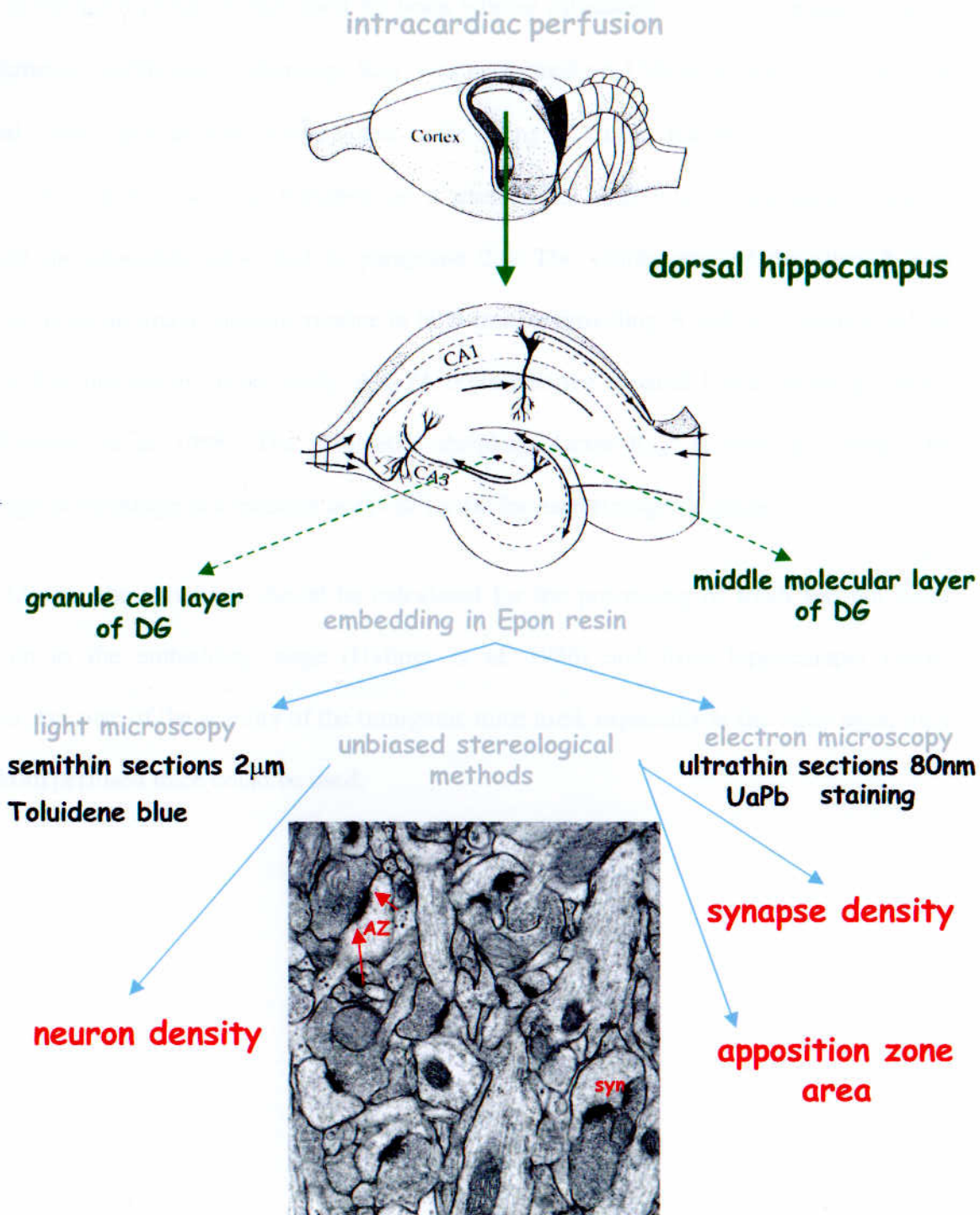


Figure 2.5 Schematic summary of the procedures used for morphometric measurements (see text for details).

## 2.6 Brain tissue shrinkage

In the same group of mice used for brain volume estimation (Table 5) measurement of the volumetric coefficient of shrinkage  $K_{vol}^3$  was performed on 150 $\mu$ m sections. One piece of temporal cortex per animal was imaged twice using a Umax flat-bed scanner: first in (chemically) 'fixed' state and mounted on a glass slide, then when embedded in Epon, following the procedure described in paragraph 2.4. The visible area of the slice A was measured using an image analysis routine in NIH Image, providing A and  $A_{EM}$  values, before and after EM processing respectively.  $A_{EM} / A$  represents the squared linear shrinkage factor  $K_{vol}^2$  (Rusakov et al. 1998). The volumetric shrinkage factor  $K_{vol}^3$  is then calculated. The percentage of shrinkage is expressed as  $(1 - K^3) \times 100$  for each transgenic group.

Ideally, the shrinkage should be calculated for the processing of fresh and not fixed tissue, up to the embedding stage (Uylings et al. 1986) and from hippocampal tissue. However, because of the scarcity of the transgenic mice used, especially at the older ages, only tissue from perfused mice could be used.

## **2.7 Choice of ratio estimates for final statistical analysis**

To avoid bias of results due to differential shrinkage related to age (Haug 1985) or ApoE genotype, it was considered relevant to take into account only measurements that were independent of volume (see explanation in Results, section 3.3). Whereas densities are estimated per unit volume, ratios of densities eliminate the volume factor for objects counted in the same tissue. To be able to interpret the data in a bias free fashion, statistical analysis was performed with synapse quantitation expressed as  $\text{Syn}/\text{nrn}$ , and synapse size expressed as  $\text{Mean AZ}/\text{syn}$  (Geinisman et al. 1992, Mayhew 1996). It is important to note that ideally, the final results of any morphometric study should relate to each animal (Howard 1998). This implies that the final measurements should be the total number of synapses and total AZ area per DG per mouse. In theory, these values could be obtained by multiplying densities by the volume of reference where these have been measured, i.e. volume of the DG. However, in practice in this study, the limited availability of mice and the geographical location of the suppliers prevented the application of this principle. Nevertheless, ratios have been used in a number of studies (Geinisman et al. 1991, Geinisman et al. 1992) and are widely accepted as pertinent measurements.

## **2.8 Immunocytochemistry quantitation**

### **2.8.1 Electron microscopy immunocytochemistry**

Post-embedding immunogold electron microscopy was performed on ultrathin sections from Epon embedded tissue. All the transgenic groups were treated equally in this experiment. Up to the sectioning stage, the preparation of the tissue was similar to that described in paragraph 2.4. Sections of dark gold interference colour (thickness of approximately 100 nm) were cut and collected on 50-mesh carbon/pioloform-coated copper grids. The sections were air dried for 1 to 2 days. They were placed in a Leica grid support plate and treated with a saturated aqueous solution of sodium metaperiodate for 20 minutes in a glass Petri dish at room temperature. This reaction partially etches the resin surface and removes some of the osmium from the tissue at the section surface. After 3 washes in distilled water, the plates supporting the grids were incubated in 0.01M sodium citrate buffer (pH 6) (see Appendix for solution recipe) for 10 minutes in a preheated oven at 80°C. A plastic lid covered the Petri dishes to avoid excessive evaporation. After heating, the grids were allowed to cool at room temperature for 15 minutes. The combination of treatment with sodium metaperiodate followed by heating on citrate buffer unmasks the antigens and increases immunolabelling (Stirling & Graff 1995). Following several attempts to obtain reasonable labelling, this method was chosen over conventional etching with sodium metaperiodate and sodium borohydride. After 3 washes in distilled water, the grids were taken off the trays and placed on top of 20µl drops in a Parafilm-lined box for all the following steps at room temperature. Non specific antigen binding was blocked with a microfiltered solution of 5% bovine albumin serum (Sigma A7030) and 2% normal goat serum (Sigma) in PBSA (BSA/NGS/PBSA) for 30 minutes (see Appendix for solutions recipes). The grids were then transferred to the rabbit anti-glutamate primary antibody solution (Sigma, UK) at 1:100 in 5% bovine serum albumin in PBSA (BSA/PBSA) for 1 hour, and washed 3 times for 5 minutes in BSA/PBSA. They



were then incubated for 1 hour in goat anti-rabbit IgG conjugated to 15 nm gold (British Biocell International, UK) diluted 1:100 in BSA/PBSA. After 2 washings of 5 minutes in BSA/PBSA, the grids were finally incubated in 0.1% aqueous glutaraldehyde solution for 5 minutes to prevent the removal of gold conjugate during staining. The sections were then air-dried and stained with uranyl acetate and lead citrate in a LKB ultrastainer. The specificity of the reaction was assessed in several ways. Firstly, the primary antibody dilution of 1:100 and the addition of 2% NGS in the first step were chosen after pilot experiments, so that there was no labelling in the lumen of vessels after incubation with both primary and secondary antibodies. Secondly, control grids were incubated with either normal rabbit serum at 1:100, or with no primary antibody, and were processed in parallel throughout the whole immunocytochemical reaction.

## **2.8.2 Quantification of the ICC reaction**

10 micrographs per animal were acquired along the axis of the cell layer, in the MML, from a JEM 1010 electron microscope at a magnification of x 12000. Negatives were scanned with an Agfa flat-bed scanner to produce digital images for the analysis. The density of gold particles was assessed in presynaptic boutons and in dendritic processes. Presynaptic boutons were identified by the presence of both pre- and post- synaptic density, with an associated cloud of synaptic vesicles in the presynaptic bouton. No attempt was made to distinguish synapses of symmetrical and asymmetrical types, or to count only gold particles that were contained in synaptic vesicles. Dendrites were identified by the presence of microtubules.

The outline of the compartment of interest (bouton or dendrite) was drawn and its area measured with a measuring tool in NIH Image software. The number of gold particles was counted in each compartment. All the compartments that contained 1 or more gold particles were measured. Mitochondrial profiles were excluded from both area and gold particle

measurements. The sum of all the counted gold particles was divided by the sum of all the measured areas to provide the number of gold particles per square micrometer of presynaptic bouton or dendrite for each mouse. Both measurements were averaged for each ApoE transgenic group.

## **2.9 Statistical analysis.**

Data were evaluated with the statistical package Statistica (version 5). Estimated synaptic (shaft, spine and total) densities, neuron density, total AZ area per unit volume, mean AZ area per synapse, hippocampal and brain volumes, were calculated for each mouse. Values from individual mice were averaged to obtain the group mean  $\pm$  standard error of the mean (SEM), for various age- and ApoE genotype- groups (group profiles shown in Table 4).

An analysis of covariance (ANCOVA) helped evaluate the effect of ApoE genotype or the effect of age on the synaptic morphological parameters. Since there were small age differences within individual groups, age was always taken as a covariate to match groups that were compared. Finally, differences between groups of age or ApoE genotype were detected by a least square difference (LSD) test as post-hoc analysis.

The level of significance was taken as  $P < 0.05$ .

## **Chapter 3 RESULTS**

### **3.1 Qualitative analysis of hippocampal anatomy and synaptic ultrastructure.**

#### **3.1.1 Hippocampal anatomy: light level analysis.**

The gross anatomy of the hippocampus was examined from semithin sections for each group of mice (Figure 3.1). All the hippocampal fields had a normal appearance in all the groups. There was no sign of aberrant development of cell layers or fibre projections at that level of observation.

#### **3.1.2 Synaptic ultrastructure: electron microscope analysis.**

Examination of electron micrographs (Figure 3.2 and Figure 3.3) taken in the MML of the DG showed that there were no obvious synaptic alterations, similar to those described in the brain of AD patients (Masliah et al. 1989, Masliah et al. 1991b), in any line of transgenic mice at any age. The synaptic terminals were generally full of numerous round and regular synaptic vesicles accompanied occasionally by normal mitochondria. There was neither enlargement of the terminals, nor the presence of multilamellar or electron dense bodies that are characteristic of AD neuropil. Microtubules in the dendrites were regularly arranged with no sign of disassembly or disruption.

The integrity of granule cells in the DG was similar in all ApoE transgenic lines (Figure 3.4). There was no evidence of neurodegeneration as indicated by either the absence of chromatin condensation in the nucleus, or absence of distention or vacuolization of the cytoplasm. In AD patients, neurofibrillary tangles are evident occasionally in the granule cells of the DG (see example of NFT in frontal cortex of AD patient in Figure 3.4). However, such neurofibrillary pathology was not observed in any of the transgenic groups.

Several markers of normal aging were present in older animals. Clusters of granules were observed in the ML of the DG as well as in CA1 (Figure 3.5), often localised around

blood vessels. At high magnification in the EM, they contained fibrillar material, almost crystalline-like, and in many cases without membrane around them. The incidence of these granules seemed to increase with age. Although they were not quantified, there was no obvious difference in their number between the different groups of mice. Such granules have been reported previously in C57BL/6 mice. They are found closely associated with astrocytic processes and are immunopositive for HSPG and laminin (Jucker et al. 1992, Jucker et al. 1994, Kuo et al. 1996). Although the functional significance of such deposits is unclear, they do not represent senile plaques with A $\beta$  deposition, and they are not related to spatial memory performance as measured in the Morris water maze (Jucker et al. 1994).

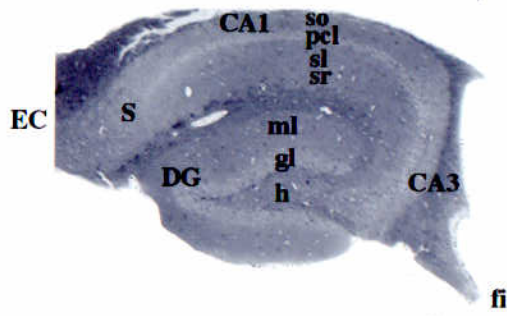
Lipofuscin intracellular deposits also appeared to increase during aging in all the groups of mice. They were localised either in astrocytic cell bodies in the neuropil of the MML or in the soma of granule cells (Figure 3.5). Lipofuscin and ceroids are 2 types of lipopigments arising from the accumulation of indigestible residues in lysosomes. They reflect the lysosomal degradative efficiency. Whereas hydrolase activity increases early in AD within late endosomes and mature lysosomes, lipofuscin is either unchanged (Mann et al. 1984) or modestly increased (Dowson 1982). It is still unclear whether this accumulation of lipopigments has adverse effects. No attempt was made to quantify this type of deposit in relation to ApoE genotype in the different groups of mice.



**WT**



**ApoE KO**



**hApoE2**



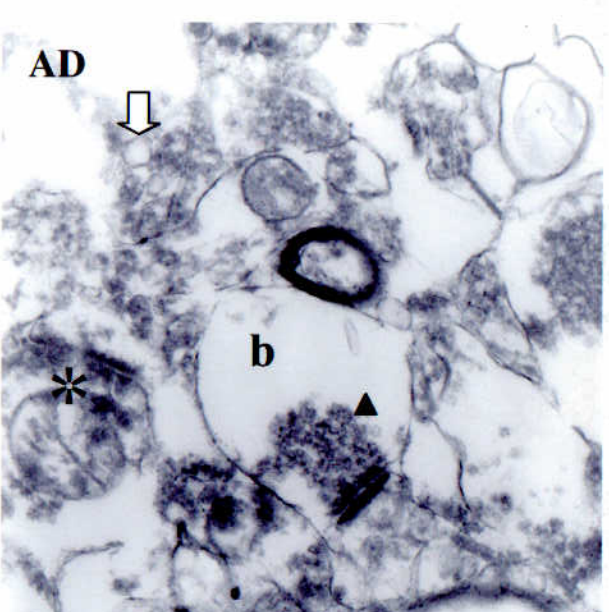
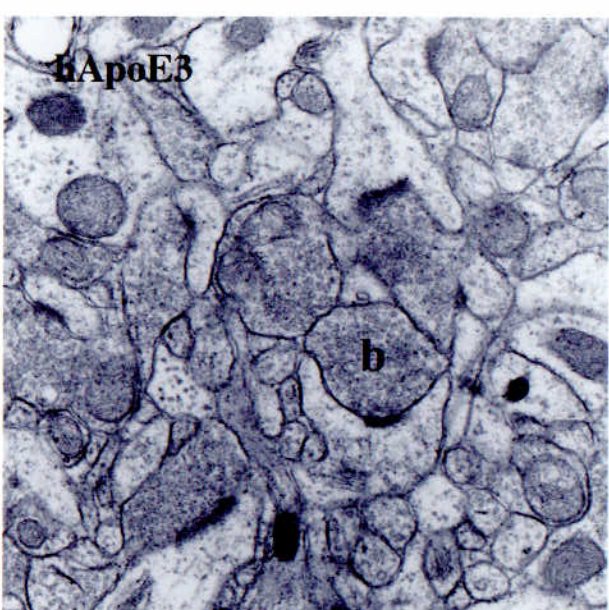
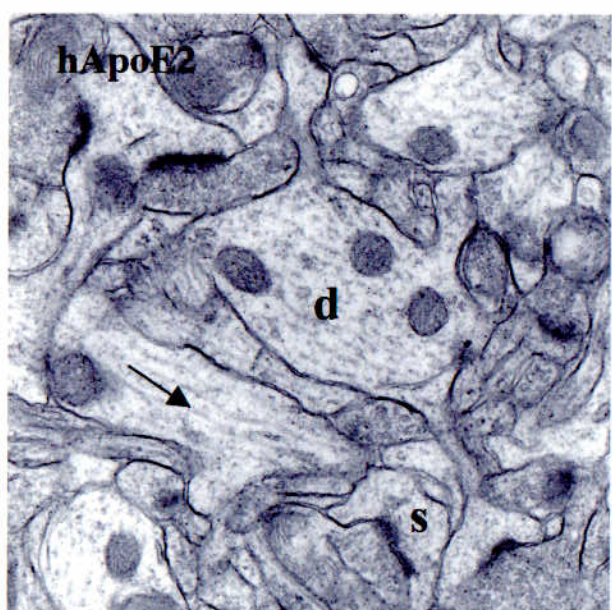
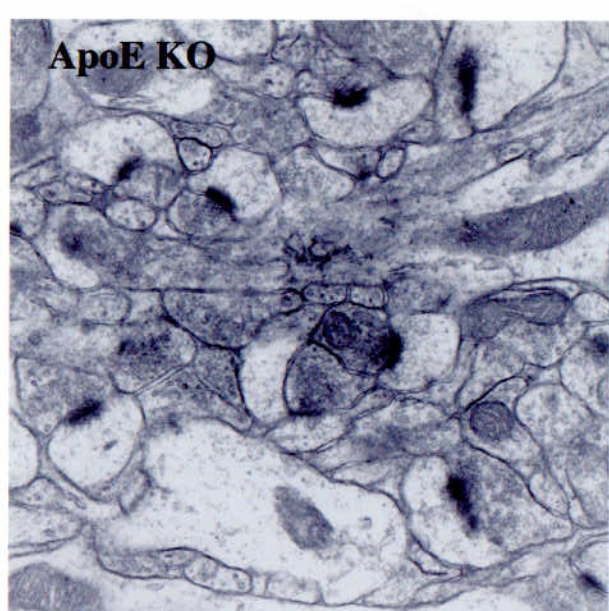
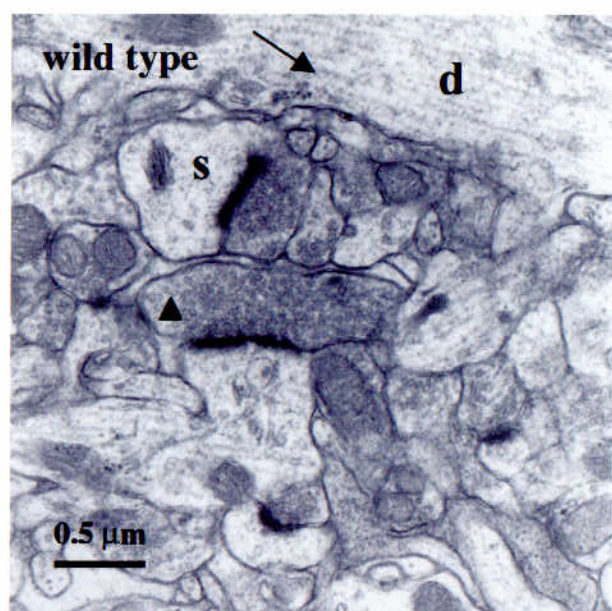
**hApoE4**



**hApoE3**

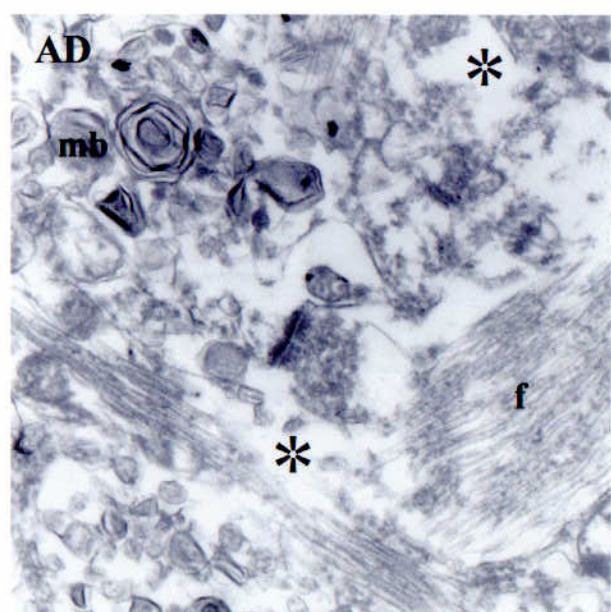
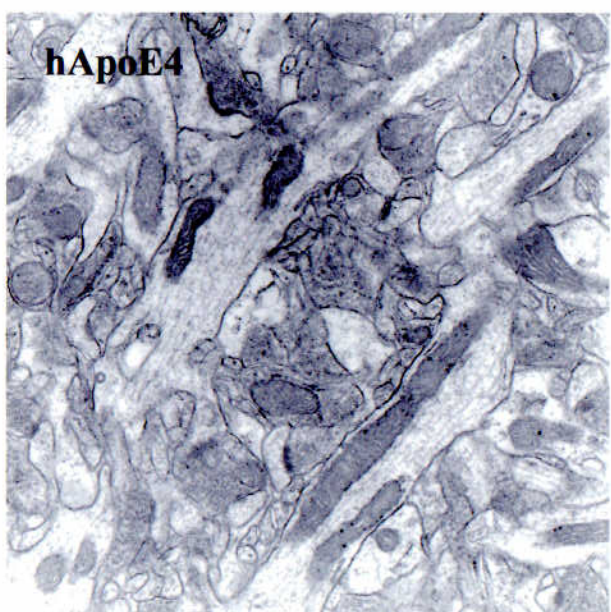
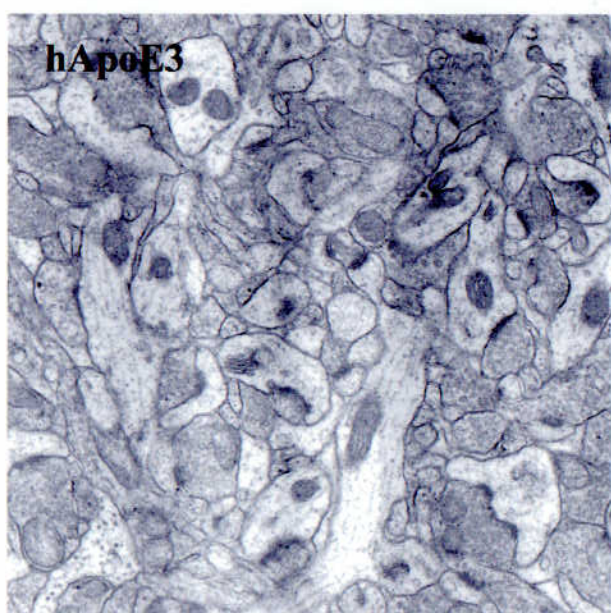
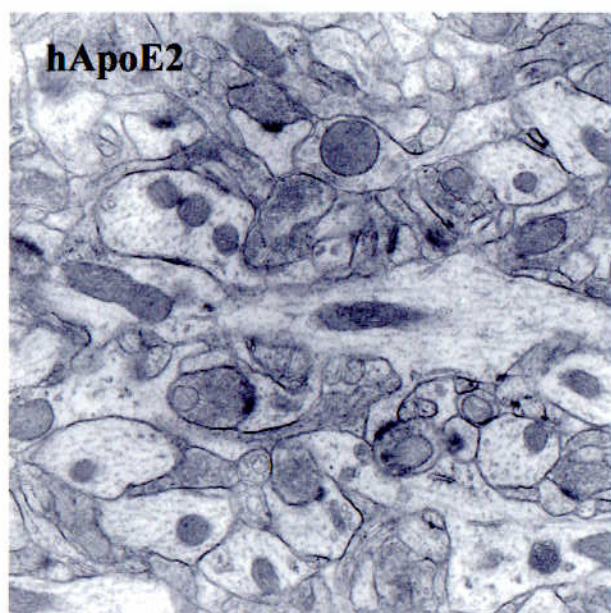
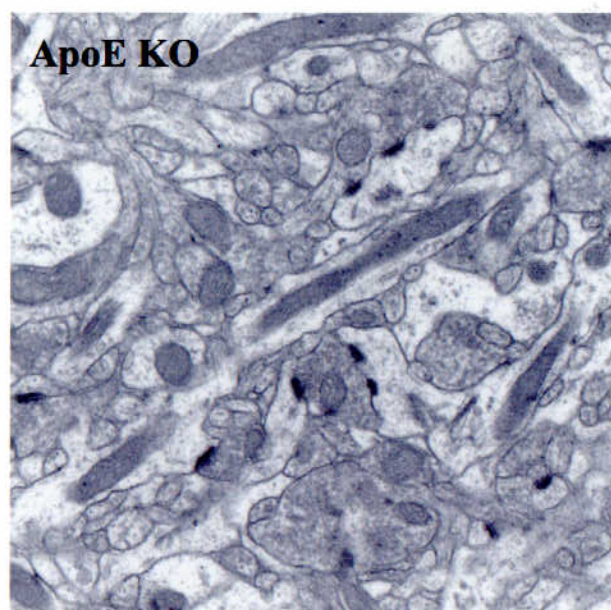
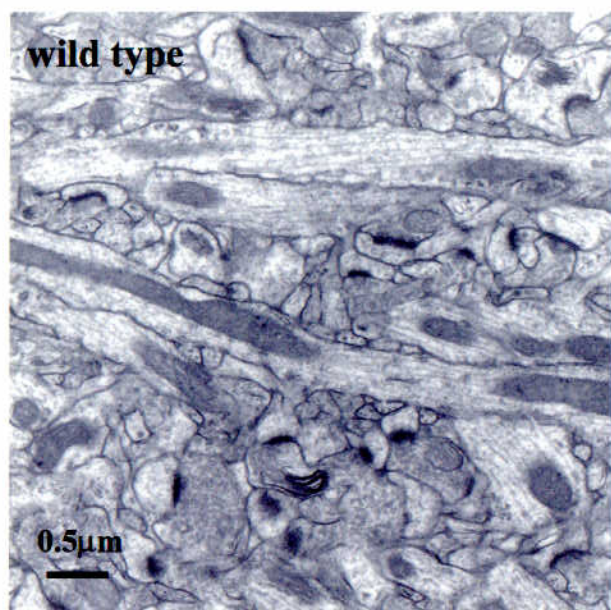
*Figure 3.1 Comparison of the gross anatomy of the hippocampus between WT, ApoE KO, hApoE2, hApoE3 and hApoE4 19 month old mice. Semithin sections were stained with toluidene blue. DG, dentate gyrus; fi, fimbria; S, subiculum; EC, entorhinal cortex; ml, molecular layer; gl, granule cell layer; h, hilus; pcl, pyramidal cell layer; so, stratum oriens; sl, stratum lucidum; sr, stratum radiatum. Scale bar is 1mm..*





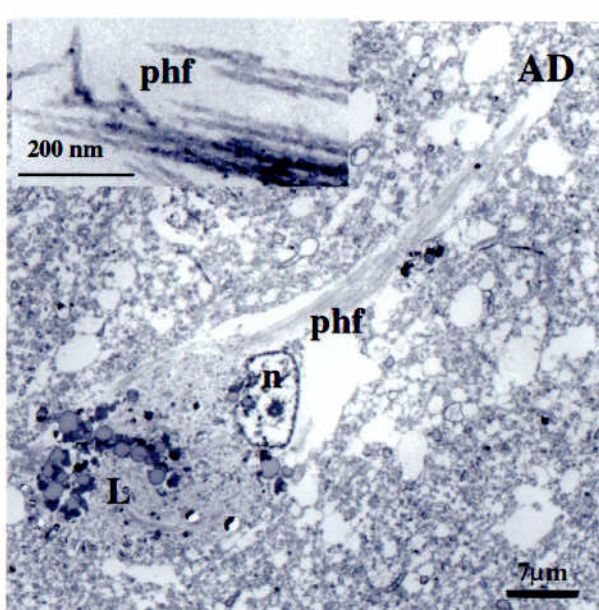
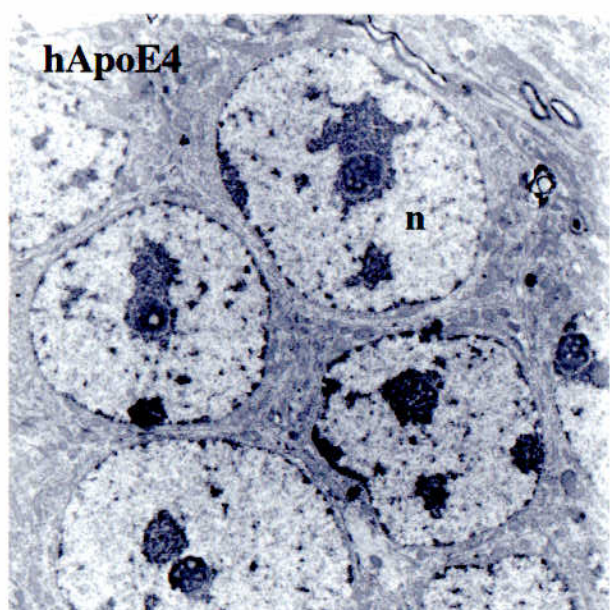
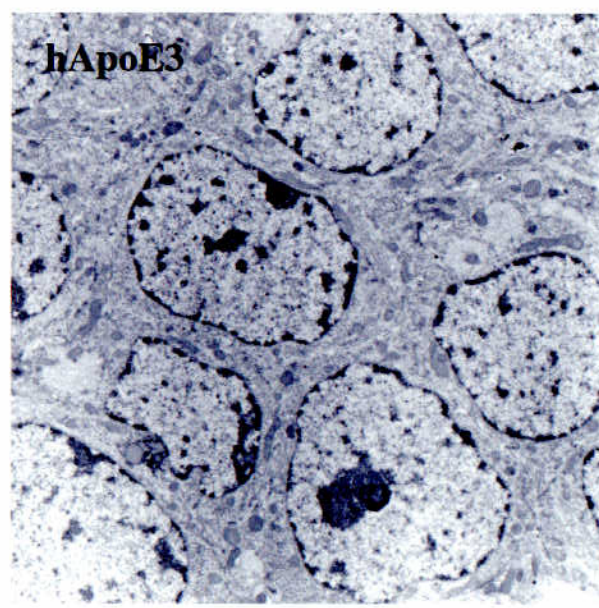
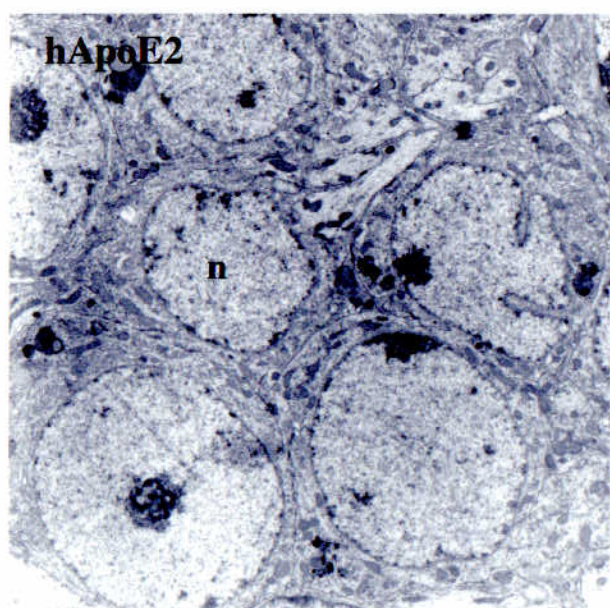
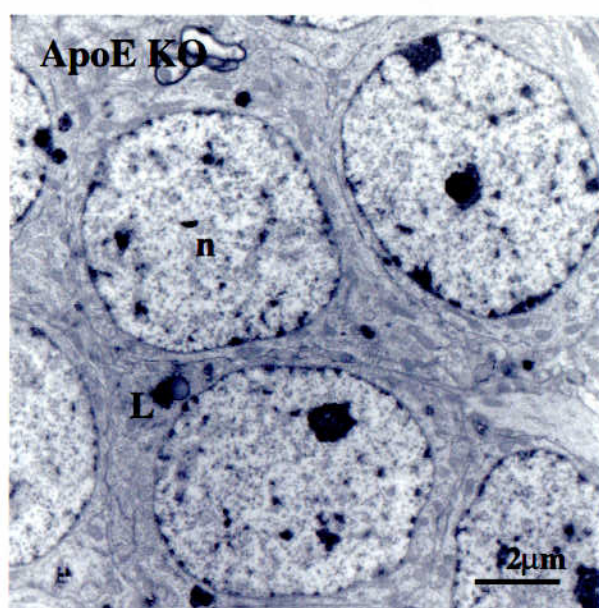
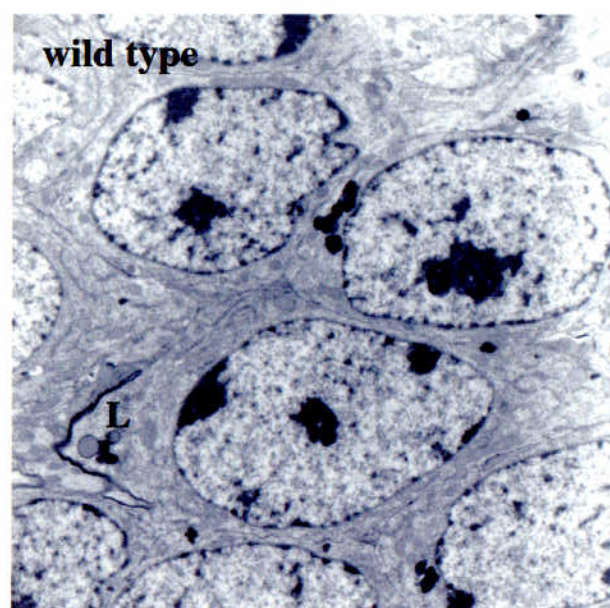
*Figure 3.2 Comparison of the synaptic ultrastructure in the neuropil. Magnification for all micrographs is x 45000. Tissue was taken from the MML of WT, ApoE KO, hApoE2, hApoE3, hApoE4 19 month old mice and from the temporal cortex of an 80 year old AD patient. Numerous synaptic contacts occur on dendritic spines (s). In all mouse groups, presynaptic boutons (b) contain abundant round synaptic vesicles (arrow heads). The microtubules (arrows) are regularly aligned in dendritic shaft (d). In contrast, in the AD brain, some presynaptic terminals are severely distended but still contain numerous vesicles. Some vesicles are clear and enlarged (open arrows). Other presynaptic terminals have lost all their synaptic vesicles (asterisk).*





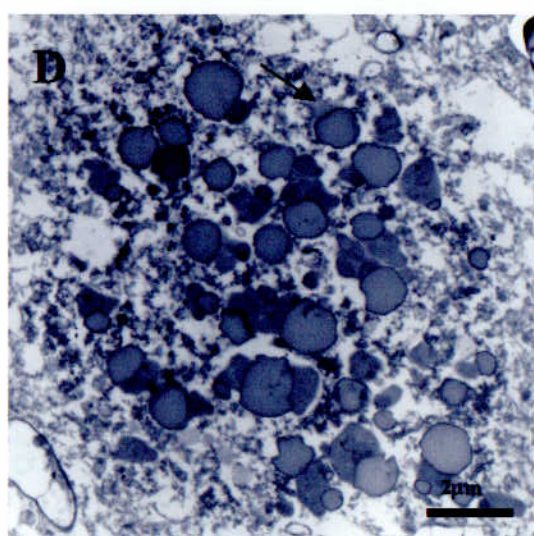
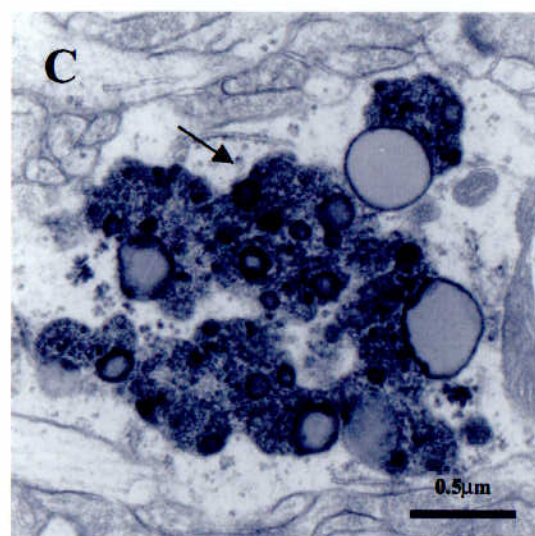
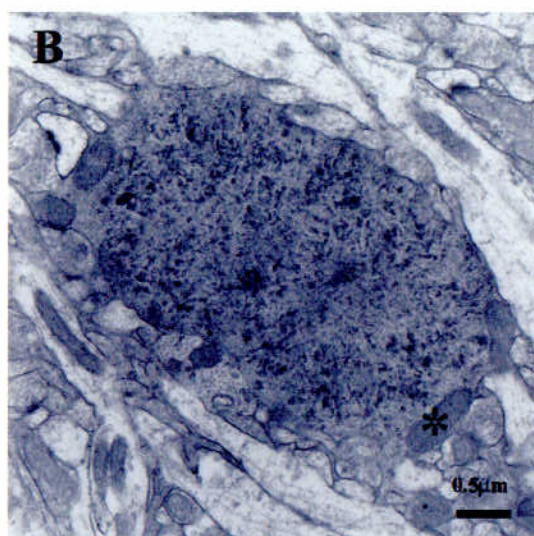
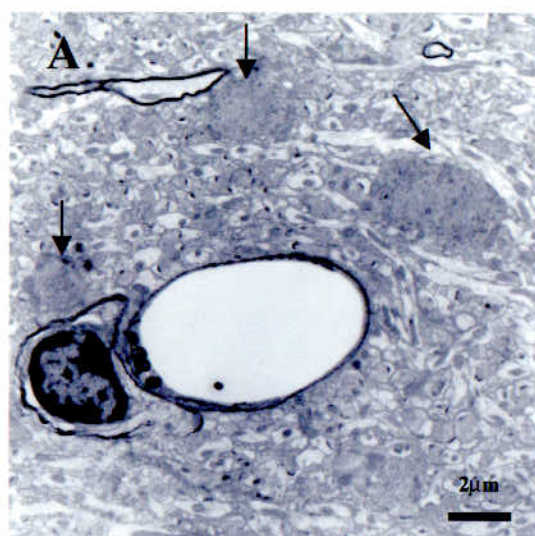
*Figure 3.3 Comparison of the synaptic ultrastructure in the neuropil. Magnification for all micrographs is  $\times 24000$ . Tissue originates from same animals (19 month old mice) and from the temporal cortex of the AD patient presented in Figure 3.2. At this lower magnification, there are obvious differences in the quality of the neuropil between any mouse group and the AD brain. Whereas the neuropil from mouse DG is tightly arranged, tissue from the AD case displays wide empty spaces (asterisk), multilamellar bodies (mb) and strands of filaments (f) likely to be paired helical filaments, which are not observed in mice. There is no sign of microtubule arrays, which may be an artefact of bad fixation typical of post-mortem samples.*





*Figure 3.4 Comparison of neuronal integrity between granule cells from WT, ApoE KO, hApoE2, hApoE3, hApoE4 19 month old mice and pyramidal cell from temporal cortex of AD patient. The cell bodies of the granule cells are densely packed in the granule cell layer, with no sign of cytoplasmic dysfunction. The nucleus is round and exhibits normal chromatin distribution in all mouse groups. Small lipofuscin (L) deposits are present in the cytoplasm. In the pyramidal neuron from the AD case, lipofuscin granules are more abundant and paired helical filaments (phf) lay in the cell body in a flame shape, forming a typical neurofibrillary tangle. The absence of neuronal membrane around the cell body and the lack of material in the nucleus (n) suggest that this neuron has reached an advanced stage of neurodegeneration.*





*Figure 3.5 Two types of deposits typically observed in the neuropil of mice. A cluster of 3 granules (A) surrounds a capillary. At higher magnification (B), the granules (arrows) represent aggregation of fibrillar material and sometimes contain mitochondria (asterisk). Synaptic contacts are not present on the membrane surrounding this inclusion and not all inclusions are bound by a membrane. C and D respectively show lipofuscin deposits (arrows) in an astrocytic process in mouse neuropil, and possibly in a degenerated cell body in the neuropil of AD patient.*



### **3.2 Influence of ApoE genotype on brain and hippocampal volumes.**

Cerebral atrophy is a macroscopic change characteristic of AD but also of human aging. Post mortem histopathological studies and magnetic resonance imaging (MRI) allowing volume measurement *in vivo*, show that the hippocampal formation is one of the earliest and most affected structures. In normal aging, hippocampal shrinkage is associated with mild memory impairment (Jobst et al. 1994, Golomb et al. 1993); and similarly in AD, atrophy of the hippocampus occurs early in patients with the mildest dementia (De Toledo Morrell et al. 1997, Jack et al. 1992). It correlates with severe neurofibrillary changes and neuronal loss but not with A $\beta$  deposition (Bobinski et al. 1996, Bobinski et al. 2000). The rate of atrophy has been estimated to be 10 times greater per year in AD compared to normal aging (Jobst et al. 1994) and some have suggested using hippocampal atrophy detected by MRI to increase diagnostic accuracy and to follow disease progression (Bobinski et al. 2000).

In the CA1 region, up to 92% of pyramidal cells are lost in the most severe AD cases. As a consequence, both cellular and dendritic layers are affected by atrophy (Bobinski et al. 1996, West et al. 1994b). The DG is affected by neurofibrillary pathology only in late-stage AD (Braak & Braak 1991) and its granular cell layer loses 25% of its volume (Bobinski et al. 1996). Furthermore, the volume of the molecular layer is decreased by 49%, probably due to the severe and early loss of its projection cells in the EC (Braak & Braak 1991, Bobinski et al. 1996, Hyman et al. 1986).

The influence of ApoE genotype on volume changes in normal aging and in AD is not clear. Some authors have reported a decreased volume of the hippocampus and the temporal lobe in  $\epsilon$ 4 AD patients (Geroldi et al. 1999, Lehtovirta et al. 1995) and in  $\epsilon$ 4 normal subjects (Tohgi et al. 1997) compared to non- $\epsilon$ 4 carriers. Others have observed no ApoE isoform-

related difference of hippocampal volume (Jack et al. 1998), and of total white matter of the brain (Azher et al. 1999). Yasuda (Yasuda et al. 1998) found a positive correlation between total brain volume and number of  $\epsilon 4$  alleles. Although methodological variations may account for these discrepancies, these data also suggest that various regions of the brain may be affected differentially by the ApoE isoforms (Geroldi et al. 1999). One recent study claimed that inheritance of the  $\epsilon 4$  allele increases the rate of brain atrophy in demented patients, as assessed by measurement of ventricular volumes, but failed to detect significant reduction of volume in the brain and temporal lobe in  $\epsilon 4$  patients (Wahlund et al. 1999).

Volumetric measurements in the brain of rodents are very rare. As yet only 2 studies have examined the effect of aging on volume changes in the rat hippocampus and described very limited changes to the DG (Rapp et al. 1999, Coleman et al. 1987). Although volumetric studies with transgenic mice used as a model for AD are currently ongoing in our laboratory, they have not yet been published (Claire Kendal, unpublished observations).

In this section, brain and hippocampal volumes were measured by Cavalieri's method in 15-19 month old hApoE2, hApoE3 and hApoE4 mice, to examine the possible influence of ApoE polymorphism on brain structure.

### **3.2.1 Results**

This study was carried out on brains of mice that were collected from DUMC in 1998. Although every effort was made to choose mice of similar age, there was a small but significant difference in age between the groups (see Table 5).

Line	n	Age (months)
hApoE2	5	18.5±0.5*
hApoE3	2	17±1
hApoE4	5	15.6±0.2*

*Table 5 Profiles of mice used for brain and hippocampal volume estimation. Mean age ±S.E.M. is significantly greater in the hApoE2 group compared to hApoE4 (p=0.0009 indicated by \*).*

Since atrophy correlates with age in humans, the 3 transgenic groups were age-matched for the following statistical analysis by using an ANCOVA with age as a covariate.

### **3.2.1.1 Brain volume**

There were no significant differences in either brain weight or volume amongst mice of different ApoE genotypes (Figure 3.6).

### **3.2.1.2 Hippocampal volume.**

Hippocampal volume in hApoE4 mice was 6% and 8% smaller in comparison to hApoE2 and hApoE3 mice respectively (Figure 3.6). However, this trend failed to reach a level of statistical significance. Combining individual genotypes into ε4 positive and ε4 negative groups (i.e. hApoE2+ hApoE3) did not change the p value to a level of significance.

Neuronal and synaptic degeneration may represent the microscopic correlate of the gross hippocampal atrophy seen during aging and AD in humans (Bobinski et al. 1996, Mizutani & Kasahara 1997, Simic et al. 1997). Therefore, a possible relationship between hippocampal volume and total synapse density, neuron density or synapse per neuron ratio in the DG, was examined in the 3 groups of ApoE transgenic mice. There was no significant correlation between hippocampal volume and these parameters within each transgenic group, or within ε4 positive and ε4 negative groups, or when all groups were pooled.

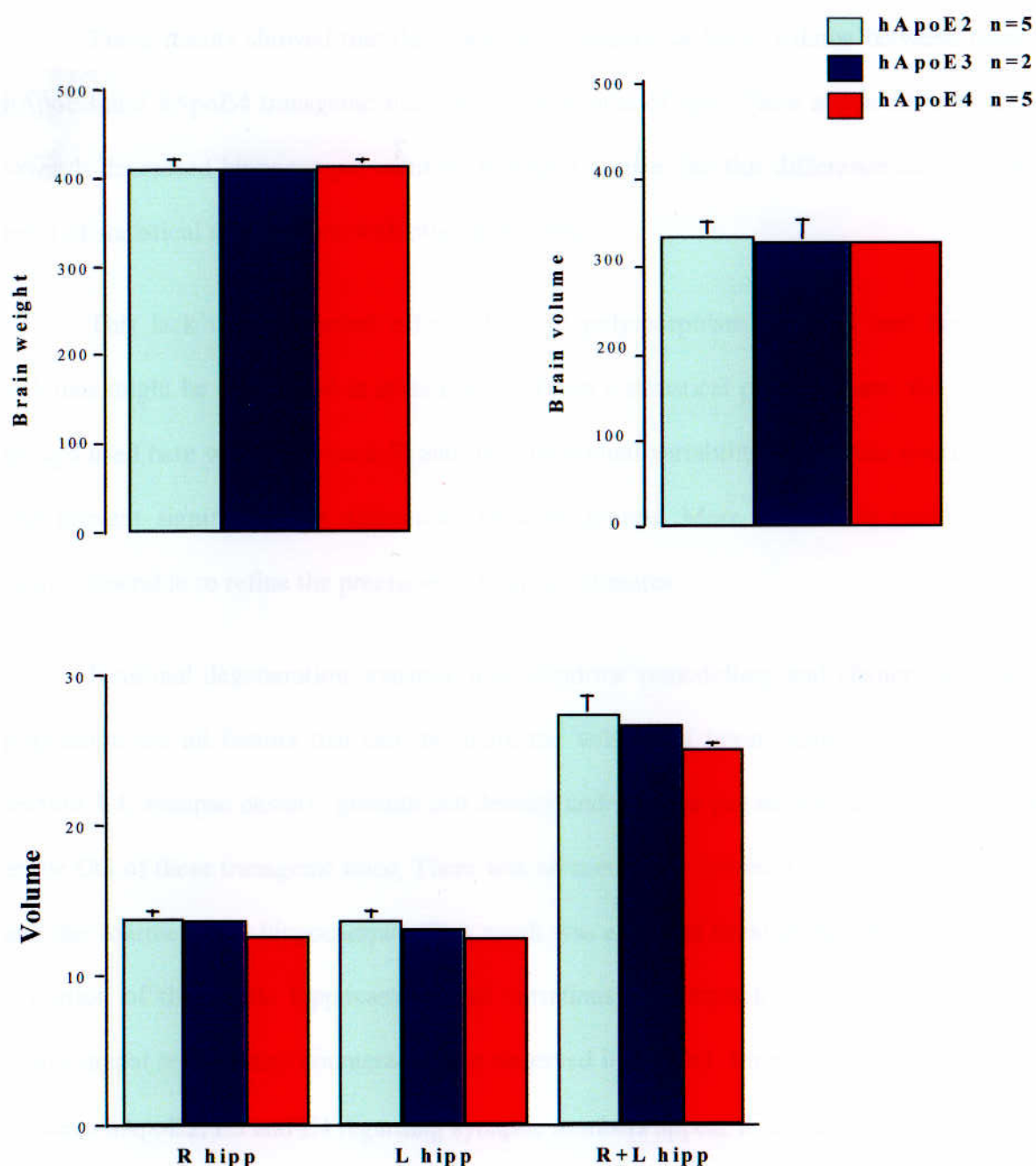


Figure 3.6 Influence of ApoE genotype on the brain and hippocampal volumes of 15-19 months old transgenic mice. Data are mean $\pm$ SEM. Brain weight is expressed per mg. Brain volume is expressed per mm<sup>3</sup>, Right (R) and left (L) hippocampal volumes per mm<sup>3</sup>. Error bars are absent when too small to be represented.

### 3.2.2 Discussion

These results showed that there was no difference in brain volume between hApoE2, hApoE3 and hApoE4 transgenic mice at 15-19 months of age. There appeared to be a trend towards decreased hippocampal volumes in hApoE4 mice, but this difference did not reach a level of statistical significance with other genotypes.

This lack of pronounced effect of ApoE polymorphism on brain and hippocampal volumes might be interpreted in several ways. From a statistical point of view, the size of the groups used here was small ( $n=2-5$ ) and inter-individual variability may cause overlap of data and prevent significance of differences between groups. More animals in each group are clearly desirable to refine the precision of volume estimates.

Neuronal degeneration, synaptic loss, dendritic remodelling and changes in glial cell population are all factors that can modulate the volume of brain tissue. As shown later in section 3.4, synapse density, granule cell density and synapse per neuron ratio were estimated in the DG of these transgenic mice. There was no correlation between any of these parameters and the volume of the hippocampus. This result was expected because the DG only represents a portion of the whole hippocampus and variations of morphological parameters in other hippocampal sectors may counteract those observed in the DG. Moreover, marked differences between hApoE2, E3 and E4 regarding synaptic numbers appear in life later than the age point chosen for this volumetric study (described in section 3.5), suggesting the possibility that the trend observed at 15-19 months of age may be accentuated at an older age.

### **3.3 ApoE genotype and tissue shrinkage**

A prerequisite to the study of morphological changes associated with aging at the EM level is to ascertain that measurements made in young and old animals are comparable. Changes in brain tissue volume during EM processing have been well described. Shrinkage of the tissue is age- dependent, i.e. brain tissue shrinks more when taken from young than old individuals (Haug 1985, Haug et al. 1984). Sources of variability in volume include mostly dehydration before resin embedding, but also the extraction of lipid constituents of membranes and any variation in content and organisation of cytoskeletal elements and organelles among cells and tissue (King 1991). Notably, ApoE is known to play a role in lipid transport (Mahley 1988) and in microtubule assembly (Nathan et al. 1995, Strittmatter et al. 1994). Although no difference in lipid composition of membranes related to ApoE genotype has yet been reported, there are indications that in humans cholesterol and phospholipid concentrations in the CSF vary with ApoE genotype (Yamauchi et al. 1999). Moreover, cholesterol distribution in synaptic membranes and phospholipid content are modified in ApoE knockout mice compared to wild type animals (Igbavboa et al. 1997, Lomnitski et al. 1999). Therefore, the possibility that tissue from the 3 ApoE isoform transgenic mice could shrink differentially was investigated.

#### **3.3.1 Results**

There was a statistically significant difference in tissue shrinkage related to the genotype of ApoE transgenic mice. Tissue taken from the temporal cortex of hApoE4 mice shrank significantly less compared to that of hApoE2 and hApoE3, which were both very similar (Table 6).

Line	%Shrinkage	n
<i>hApoE2</i>	16.2±1.5*	5
<i>hApoE3</i>	15.9±1.2**	2
<i>hApoE4</i>	9.7±0.5	5

*Table 6 Shrinkage of temporal cortex tissue during EM processing in ApoE transgenic mice. %shrinkage is the mean±S.E.M. Statistically significant differences from hApoE4 group are indicated by \* (p=0.003) and \*\* (p=0.018).*

### 3.3.2 Discussion

These results obtained from 15-19 month old mice showed that ApoE genotype is associated with different degrees of tissue shrinkage during EM processing. The reasons for this differential shrinkage are not known and such an effect has never been reported before. The lipid composition of brain tissue is likely to influence the degree of shrinkage (King 1991) and may be modulated by ApoE genotype (Igbavboa et al. 1997, Yamauchi et al. 1999), providing a possible link between these last 2 parameters. Determining the effect of ApoE polymorphism on the composition and biophysical properties of cellular membranes may help understand the role of the 3 ApoE isoforms in lipid redistribution and neuronal regeneration (Poirier et al. 1991, Poirier et al. 1993a). Whether the shrinkage values obtained here also vary with age is unknown. Haug (Haug 1985) established clearly that brain tissue shrinkage is age-dependent in normal humans. This suggests the possibility of additional age-related changes of tissue shrinkage in ApoE transgenic mice.

Taken together, these findings justify the later use of volume density ratios which are independent of volume changes, for bias-free comparison of synapse number and size between the different ApoE transgenic groups, at different ages.

### **3.4 Synaptic and neuronal morphometry in the dentate gyrus of ApoE transgenic mice.**

The aim of the following 2 sections (3.5 and 3.6) is to determine the influence of ApoE genotype on synaptic morphological changes associated with aging in the DG. Alterations in synapse number and size with aging have been reported previously in other species such as humans and rats (see Table 2 in Introduction 1.1). However, they have not yet been described in mice at the EM level, although this species is now used very commonly for aging research and for the development of transgenic models of neurodegenerative diseases.

Several morphometric parameters were estimated, using strict stereological methods, first in the brain of wild type mice to provide a basic knowledge, and then in genetically manipulated mice relevant to ApoE polymorphism.

Synaptic (shaft, spine and total) densities, granule cell density and total AZ area were first estimated in the DG of WT, ApoE KO, hApoE2, E3 and E4 mice at 3 age points (young, adult, aged). As established previously in sections 2.7 and 3.3, density measurements cannot be used as such if they are to be compared between young and old animals. Ratios of densities were therefore calculated to avoid the bias introduced by differential shrinkage.

All these measurements and calculated values are presented in Table 4. They are further described in section 3.5 which examines the influence of ApoE polymorphism on synaptic parameters during aging for mice of different genotypes (Figure 3.7-Figure 3.10), whereas section 3.6 compares these same synaptic characteristics for mice of different genotypes at each of the 3 age points (Figure 3.11-Figure 3.15)



	Number of animals	Age (months)	Total synaptic density	Neuron density	Syn/nrn ratio	Total AZ area	Mean AZ area
<b>Wild type</b>	17						
<i>young</i>	6	8	3.54 $\pm$ 0.26	0.00151 $\pm$ 0.0002	2487 $\pm$ 232	0.082 $\pm$ 0.009	0.023 $\pm$ 0.002
<i>adult</i>	5	16	2.99 $\pm$ 0.13	0.00152 $\pm$ 0.0000	1980 $\pm$ 122	0.071 $\pm$ 0.007	0.024 $\pm$ 0.002
<i>aged</i>	6	21	3.48 $\pm$ 0.15	0.00143 $\pm$ 0.0001	2599 $\pm$ 299	0.070 $\pm$ 0.004	0.020 $\pm$ 0.001
<b>ApoE KO</b>	16						
<i>young</i>	6	8	3.41 $\pm$ 0.28	0.00159 $\pm$ 0.0001	2193 $\pm$ 199	0.069 $\pm$ 0.006	0.020 $\pm$ 0.001
<i>adult</i>	4	17	3.22 $\pm$ 0.43	0.00135 $\pm$ 0.0000	2367 $\pm$ 233	0.083 $\pm$ 0.015	0.025 $\pm$ 0.002
<i>aged</i>	6	22 to 24	3.31 $\pm$ 0.18	0.00149 $\pm$ 0.0001	2293 $\pm$ 198	0.064 $\pm$ 0.004	0.019 $\pm$ 0.001
<b>hApoE2</b>	13						
<i>young</i>	4	10	2.70 $\pm$ 0.18	0.00114 $\pm$ 0.0001	2377 $\pm$ 191	0.067 $\pm$ 0.006	0.025 $\pm$ 0.001
<i>adult</i>	3	14 to 16.5	3.47 $\pm$ 0.62	0.00107 $\pm$ 0.0001	3222 $\pm$ 467	0.070 $\pm$ 0.011	0.020 $\pm$ 0.001
<i>aged</i>	6	19 to 22	3.69 $\pm$ 0.38	0.00139 $\pm$ 0.0001	2765 $\pm$ 321	0.073 $\pm$ 0.008	0.020 $\pm$ 0.001
<b>hApoE3</b>	7						
<i>young</i>	0	—	—	—	—	—	—
<i>adult</i>	7	14 to 18	2.58 $\pm$ 0.15	0.00136 $\pm$ 0.0001	1926 $\pm$ 161	0.054 $\pm$ 0.005	0.021 $\pm$ 0.001
<i>aged</i>	0	—	—	—	—	—	—
<b>hApoE4</b>	23						
<i>young</i>	6	6	2.89 $\pm$ 0.28	0.0010 $\pm$ 0.0001	3070 $\pm$ 320	0.052 $\pm$ 0.006	0.018 $\pm$ 0.001
<i>adult</i>	11	15 to 17	2.99 $\pm$ 0.20	0.0011 $\pm$ 0.0001	2872 $\pm$ 261	0.061 $\pm$ 0.005	0.020 $\pm$ 0.001
<i>aged</i>	6	19 to 20	3.34 $\pm$ 0.12	0.0017 $\pm$ 0.0001	2027 $\pm$ 177	0.072 $\pm$ 0.005	0.022 $\pm$ 0.001

Table 7 Morphometric measurements in the DG of ApoE transgenic mice. Synapse density is expressed in synapse per  $\mu\text{m}^3$ , neuron density in neuron per  $\mu\text{m}^3$ , total AZ area in  $\mu\text{m}^2$  per  $\mu\text{m}^3$ , mean AZ area in  $\mu\text{m}^2$  per synapse. Data are mean  $\pm$ SEM. Only Syn/nrn and mean AZ/syn ratios (in red) were included in the final statistical analysis for their comparison. Values printed in green were estimated by Heather Davies

## **3.5 Synaptic plasticity associated with aging varies with ApoE genotype.**

### **3.5.1 Results**

#### **3.5.1.1 *Wild Type mice***

C57BL/6 mice are the most commonly used strain in neuroscience and aging research today. They perform well in tests of memory and cognition such as the Morris water maze (Gerlai 1996b, Owen et al. 1997, Rouillet & Lassalle 1995). They also are good breeders and do not exhibit any particular neurological and anatomical defects, as opposed to other inbred strains of mice (reviewed in Gerlai 1996b, Lathe 1996). Therefore, they provide a good genetic background to study the effect of a missing gene in KO animals, or the effect of an added gene in transgenic animals. However, very few studies have characterised age-related morphological changes in the brain of this strain (Calhoun et al. 1998a, Ingram & Jucker 1999, Jucker et al. 1992, Jucker et al. 1994, Kuo et al. 1996) despite its use in transgenic models of neurodegenerative diseases. Here, the synaptic morphological changes associated with aging in the DG of C57BL/6 mice are described, using unbiased stereological tools at the EM level.

These data are provided as controls for ApoE KO mice (section 3.6). Whereas the spine Syn/nn ratio did not vary significantly with age (Figure 3.7), shaft synapses notably decreased in number between young and adult age (-64%) until older age (-50%). Since shaft synapses only represent a small proportion of all the synapses in the MML of DG (4 to 9%), their variation did not affect the total Syn/nn ratio, which showed a similar pattern of changes as the spine Syn/nn ratio. Syn/nn ratio for the adult group was 20% and 24% smaller compared to young and aged groups respectively but these variations were not significant. The mean AZ/syn remained stable until the old age. In conclusion, WT mice showed no significant age-related changes in either overall synapse per neuron ratio or mean AZ/syn ratio.

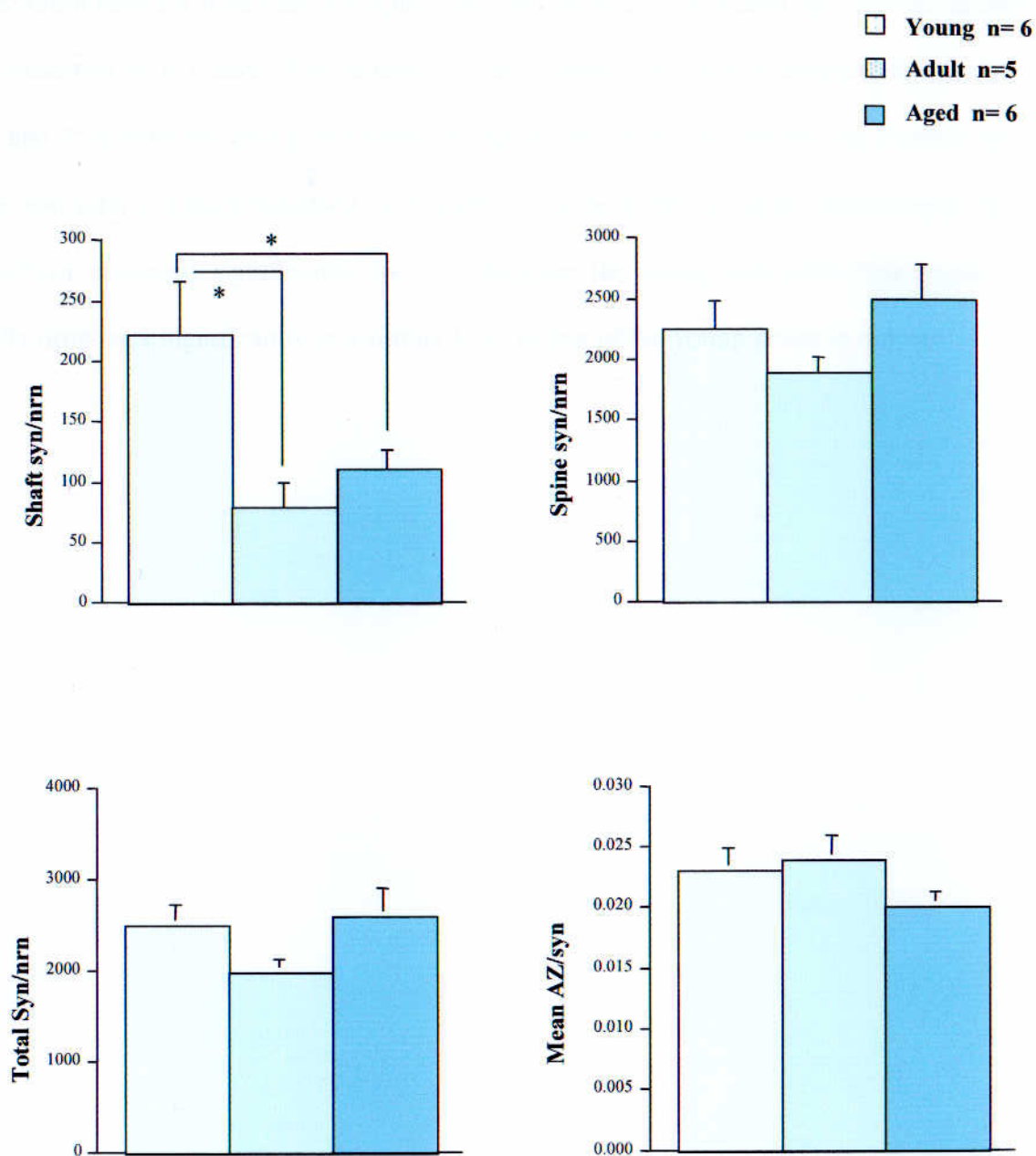


Figure 3.7 Synaptic changes in the MML of DG associated with aging for WT mice. Mean AZ/syn is expressed as  $\mu\text{m}^2$  per synapse. Data are mean $\pm$ SEM. Statistically significant differences are indicated by asterisks ( $p<0.05$ ).

#### **3.5.1.2 *ApoE KO mice***

Syn/nrn ratio for both shaft and spine synapses showed very similar age-related changes to those observed in WT mice. The number of shaft synapses per neuron dropped significantly by 52% and 55% between young and adult/ old age respectively. In contrast, the Syn/nrn ratio for spine and total synapses remained very stable throughout the age span. Surprisingly, their mean AZ/syn increased significantly by 25% between the young and adult time points to eventually drop back significantly to a similar level to that of the young group in old age.

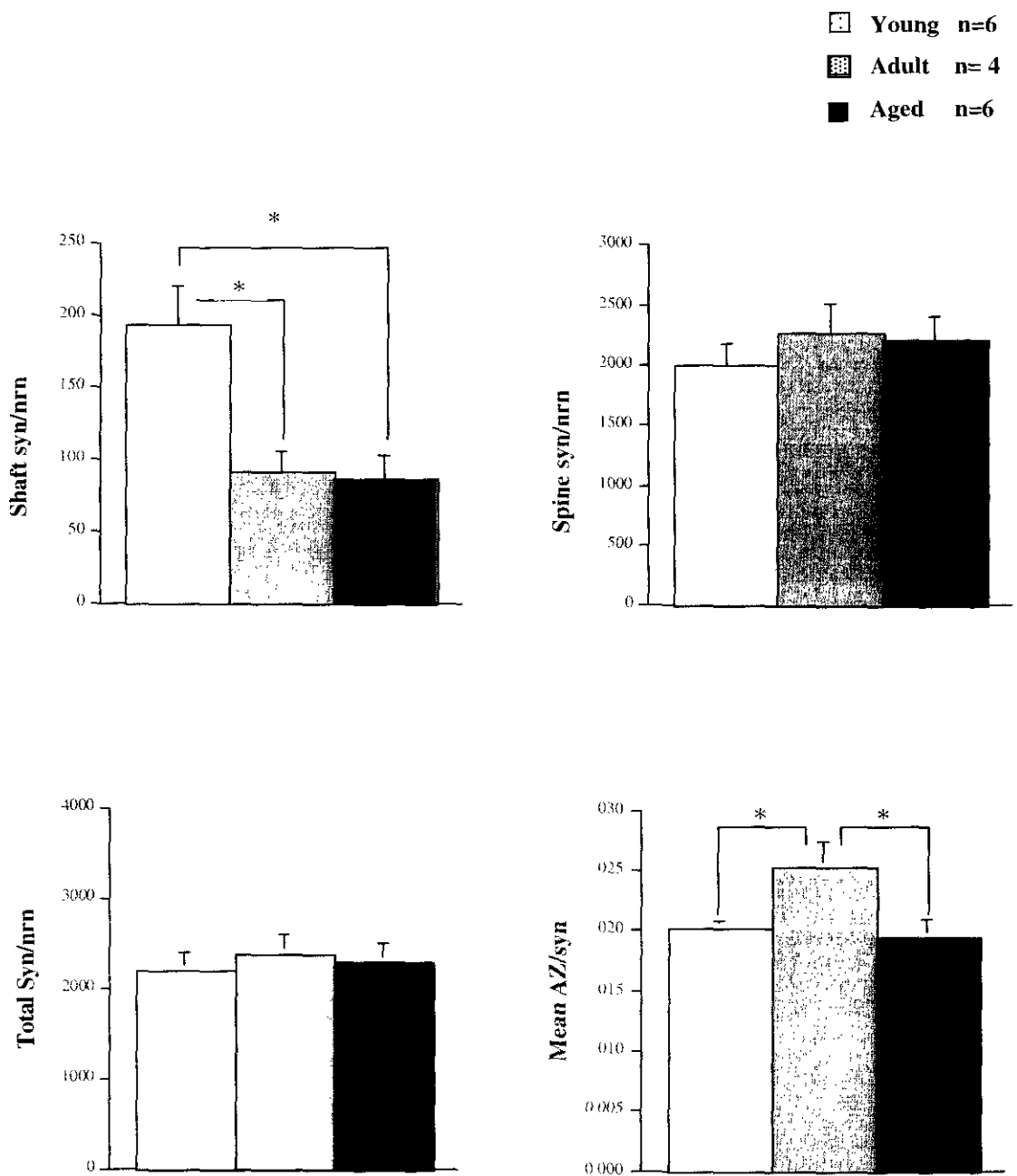


Figure 3.8 Synaptic changes in the MML of DG associated with aging for ApoE KO mice. Mean AZ/syn is expressed as  $\mu\text{m}^2$  per synapse. Data are mean  $\pm$  SEM. Statistically significant differences are indicated by asterisks ( $p < 0.05$ ).

### **3.5.1.3 *hApoE2 mice***

None of the categories of synapses was affected by aging, as indicated by their unchanging Syn/nrn ratio across ages. The mean AZ area per synapse, however, decreased steadily, falling by 20% in aged compared to young mice. Notably, mean AZ/syn correlated negatively with age ( $r=0.68$ ,  $p=0.01$ ) in the whole sample of hApoE2 mice ( $n=13$ ).



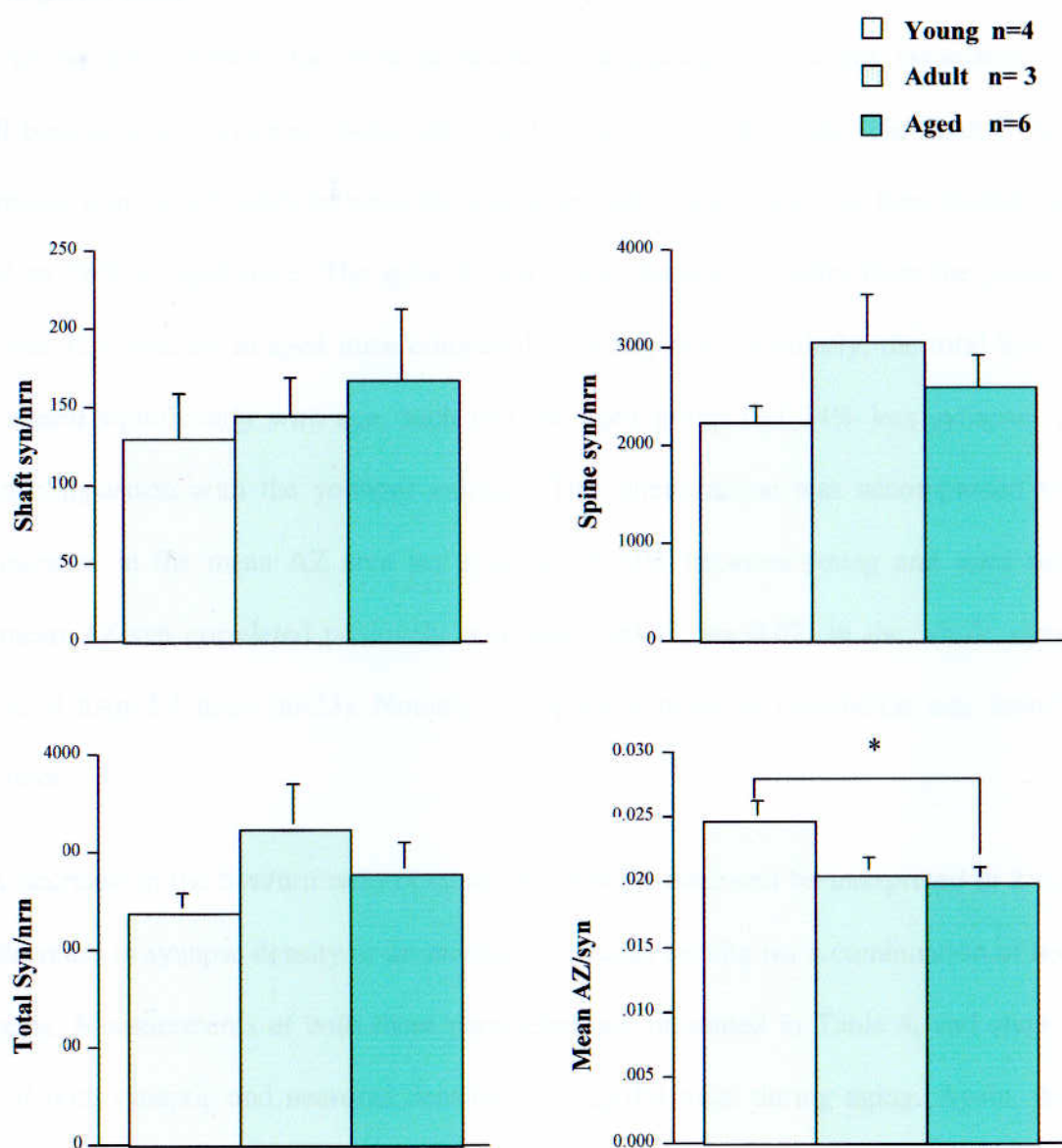


Figure 3.9 Synaptic changes in the MML of DG associated with aging for hApoE2 transgenic mice. Mean AZ/syn is expressed as  $\mu\text{m}^2$  per synapse. Data are mean  $\pm$  SEM. Statistically significant differences are indicated by asterisks ( $p < 0.05$ ).

#### **3.5.1.4 hApoE4 mice**

hApoE4 mice showed the most pronounced morphological changes associated with aging; all populations of synapses were affected, both in number and size. The shaft Syn/nrn ratio increased non-significantly between the young and adult time points but then dramatically decreased by 78% in aged mice. The spine Syn/nrn ratio declined steadily from the youngest age and was 32% smaller in aged mice compared to young ones. Similarly, the total Syn/nrn ratio decreased significantly with age, such that the aged group had 34% less synapses per neuron in comparison with the younger animals. This phenomenon was accompanied by a parallel increase in the mean AZ area per synapse of 22% between young and aged mice. Indeed, mean AZ/syn correlated positively with age ( $r=0.47$ ,  $p= 0.02$ ) in the whole sample population of hApoE4 mice ( $n=23$ ). Notably, an opposite negative correlation was found in hApoE2 mice.

A decrease in the Syn/nrn ratio observed in hApoE4 mice can be interpreted in 2 main ways: a decrease in synapse density or an increase in neuron density (or a combination of both) during aging. Measurements of both these parameters are presented in Table 4, and show an increase of both synaptic and neuronal densities in hApoE4 mice during aging. Again, these raw values should be considered with caution because they are not corrected for shrinkage at the 3 age points. Although the increase in neuron density suggests that it is the cause for the decrease of the Syn/nrn ratio, additional measurements ruled out this possibility. The thickness of the granule cell layer was measured at the 3 age points (Table 8). In aged animals, the granule cell layer was significantly thinner than at earlier age points.



<b>hApoE4 mice</b>	<b>Young</b>	<b>Adult</b>	<b>Aged</b>
<b>Thickness of granule cells layer (<math>\mu\text{m}</math>)</b>	68.6 $\pm$ 5.2	74.1 $\pm$ 3.7*	55.9 $\pm$ 4.1*
<b>Granule cell density per <math>\mu\text{m}^3</math></b>	0.0010 $\pm$ 0.0001	0.0011 $\pm$ 0.0001	0.0017 $\pm$ 0.0001
<b>n</b>	6	11	6

*Table 8 Changes of thickness of the granule cell layer and neuron density during aging in hApoE4 mice. Data are mean  $\pm$  SEM. Statistically significant difference for granule cell thickness was determined by ANCOVA (age as a covariate) and is indicated by \* ( $p<0.05$ ).*

This implies that tissue from aged hApoE4 mice shrinks more (either *in vivo* or during processing) than tissue from adult and young mice, and indicates that the increase in neuron density is the result of shrinkage rather than increase in actual neuronal numbers. Therefore, the decrease in Syn/nrn ratio can be interpreted mainly as a decrease in synapse numbers.

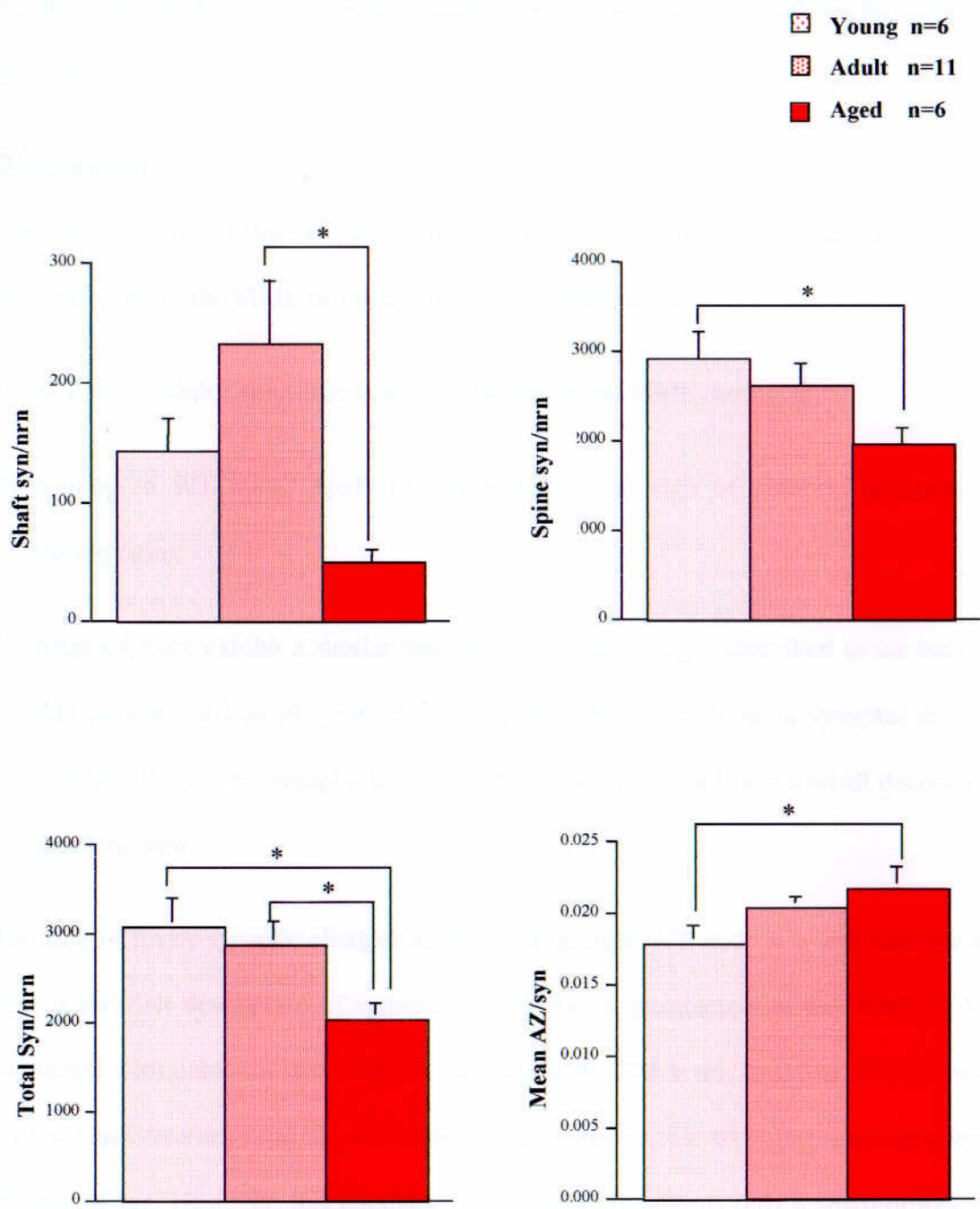


Figure 3.10 Synaptic changes in the MML of DG associated with aging for hApoE4 transgenic mice. Mean AZ/syn is expressed as  $\mu\text{m}^2$  per synapse. Data are mean  $\pm$  SEM. Statistically significant differences are indicated by asterisks ( $p < 0.05$ ).

#### **3.5.1.5 *hApoE3 mice***

As only adult hApoE3 mice were available, the effect of aging could not be examined for this genotype.

### **3.5.2 Discussion**

The main finding of this section is that ApoE genotype influences synaptic plasticity associated with aging in the MML of mice. Three main points arose:

- 1) WT mice exhibit very little synaptic changes in the MML during aging.
- 2) Similar to WT mice, ApoE KO mice display no sign of synaptic degeneration during aging.
- 3) hApoE4 mice exhibit a similar pattern of synaptic changes described in the brain of AD patients: a loss of synapses accompanied by an increase in synaptic area. In contrast, there is no synaptic loss in aged hApoE2 mice and only a small decrease in synaptic area.

The lack of major synaptic changes in the MML in aged WT mice is worth mentioning. As yet, this is the first description of synaptic morphometric parameters in the MML of WT mice, established with unbiased stereological methods at the EM level. In C57BL/6 mice used here, only the shaft Syn/nn ratio shows a dramatic and irreversible 64% decrease between 8 and 16 months of age. However, this population of synapses represents only a small proportion (4 to 9%, throughout life) of the total number of synapses in the MML whereas spine synapses, which form the overwhelming majority, are not affected at all. Similarly, the mean AZ area per synapse does not vary with aging. In agreement with this lack of synaptic loss, Calhoun (Calhoun et al. 1998a) observed no change in synaptophysin positive presynaptic boutons number in DG and CA1 during aging in the same strain of mice, and using stereological

counting methods at the light microscopy level. In addition, numbers of DG granule cells and CA1 pyramidal cells were remarkably preserved. This lack of synaptic loss in aged mice was unexpected and contrasts with observations made in aged rats. Indeed, although aged rats display no loss of principal neurons in the hippocampus (Rapp & Gallagher 1996, Rasmussen et al. 1996), they lose 24% of spine synapses in the MML and the IML whereas, in contrast to C57BL/6 mice, their shaft synapse numbers do not vary significantly (Geinisman et al. 1992, Smith et al. 1999). In both aged monkeys (Tigges et al. 1995, Tigges et al. 1996), and aged humans (Hamos et al. 1989, Lippa et al. 1992, Scheff et al. 1996, Scheff & Price 1998), synapse density is not altered significantly in the DG. This illustrates great species differences in response to aging (Table 2). However, it is well known from several studies in different species other than mice, that some individuals do not show any significant cognitive or memory impairment associated with aging and can be seen as healthy elderly, whereas others develop severe deficits (Rapp & Amaral 1992). Therefore, it cannot be ruled out that the lack of synaptic loss observed in mice in this study may be due to a cluster of 'healthy aging' individuals, that may not be a representative sample of aging in mice. Morphological studies combined with behavioural tests could help resolve this issue. Alternatively, mice from the 'aged' group (24 months old) may not be old enough to display evident synaptic changes, the mean life span of C57BL/6 strain being 26-28 months (Jucker & Ingram 1997). Obviously, these points also apply to the other groups of mice included in this study. It is not known if unhealthy animals were removed from breeding colonies, or if ApoE genotype influences mortality rate and life expectancy in human ApoE transgenic mice.

ApoE KO mice failed to show any sign of synaptic deficit during aging, as indicated by an almost identical pattern of changes to WT mice. The only difference with this latter group was a transient increase in mean synaptic area at 17 months of age. The reasons for this effect are unclear, and there are no physiological and behavioural data available to correlate with it.

One likely explanation could be the fact that mice from the young and aged groups and those of the adult group were bred in 2 different places (DUMC, NC, USA and Glaxo-Wellcome, Stevenage, UK). Although the breeding conditions were almost identical, uncontrollable factors may have influenced this synaptic parameter. Mice from both sources were back-crossed at least 6 times to WT animals to reduce possible influence of genetic background (see section 2.1 for detailed explanation); however, the exact number of back-crossings was not known and different numbers may have introduced the small variability observed in these results. Two studies using other ApoE KO transgenic lines have described up to 40 % loss of synaptophysin -positive presynaptic terminals and MAP2 positive neuronal dendrites in the whole hippocampus from 3 to 20 months of age (Buttini et al. 1999, Masliah et al. 1995). In contrast, authors using the same ApoE KO line as here found no difference in these same measurements and in the density of cholinergic fibres in the hippocampus, in mice as old as 24 months (Fagan et al. 1998, Gandy et al. 1995, Parker et al. 1996). Clearly, these data suggest that, in addition to measurement procedures, the nature of the transgenic line may be a crucial factor of variability of results between laboratories. For example, transgene construct, background strain and number of back-crossings may all contribute to the observed phenotype. Since other groups have described age-dependent degeneration in the brain of some lines of ApoE KO mice, it seems unreasonable to conclude that ApoE is not involved in brain plasticity. More likely, compensatory factors may account for the results presented here (Crawley 1996, Gerlai 1996b, Gerlai 1996a, Lathe 1996). Like ApoE, both ApoD and ApoJ expression increases in the DG after ECL (Bertrand et al. 1995, May et al. 1990, Terrisse et al. 1998), suggesting that these lipoproteins could compensate for the lack of ApoE for neuronal repair.

In human transgenic mice, synaptic morphometric parameters were altered in very distinct ways, depending on ApoE genotype. Whereas hApoE2 mice displayed no synaptic loss and a slight decrease in synaptic size during aging, hApoE4 mice lost one third of all their

synapses between the young and the aged time-points. Notably, the average synaptic size increased with age. Two main conclusions can be drawn from these data: 1) the expression of the hApoE2 isoform on an ApoE KO background in hApoE2 mice appears to maintain the structural synaptic connectivity during aging, and prevents the important loss of shaft synapses observed in ApoE KO and WT mice; 2) the expression of the hApoE4 isoform on an ApoE KO background in hApoE4 mice produces a pattern of synaptic changes remarkably different from that observed in WT, ApoE KO and hApoE2 mice. If compensatory factors were maintaining the number of synapses in aged ApoE KO mice to prevent neurodegeneration, it is clear that they are not sufficient to counter the effect of the hApoE4 isoform on synaptic plasticity. In agreement with these results, Buttini et al (Buttini et al. 1999) reported a significant loss of synaptophysin positive presynaptic terminals and MAP2-positive neuronal dendrites in a different line of human ApoE4 transgenic mice between the age of 3-4 months and 7-9 months (Buttini et al. 1999). The counterbalance of synaptic size against number in hApoE4 mice here is very similar to that which Scheff and others described in the OML of the DG (Scheff et al. 1996) and other cortical areas (Adams 1987, Bertoni Freddari et al. 1986, Bertoni Freddari et al. 1990, Bertoni Freddari et al. 1996b, Bertoni Freddari et al. 1996a, DeKosky & Scheff 1990, Scheff et al. 1990, Scheff & Price 1993, Scheff & Price 1998) of elderly and AD patients. The fact that the total AZ area is conserved in these cases suggested that the increase in mean AZ area reflects an enlargement of the remaining synapses to compensate synaptic loss and maintain a level of synaptic transmission. Whether the 22% increase in synaptic size detected here is sufficient enough to compensate 34% in synaptic loss is an important issue with regard to the functional consequences of such a loss. The total AZ area was calculated here for each group. However, this parameter is expressed per unit volume and is therefore biased by tissue shrinkage specific to each age and ApoE genotype. Therefore only total AZ area corrected for individual shrinkage allows the comparison of old and young mice with different ApoE

genotype. Unfortunately, we were unable to calculate the shrinkage for all of the 76 animals and it would be unwise to draw any conclusion for uncorrected data. If antibodies directed towards synaptic protein are markers of all synapses, it is reasonable to believe that total compensation of synaptic loss by increase of synaptic size would result in no difference in immunolabelling between young and old animals. Buttini's immunocytochemical study (Buttini et al. 1999) indicates that this is not the case between 4 and 9 month old mice. In addition, in the frontal cortex of AD patients, up to an approximately 35% decrease in synaptic density is compensated by increase in mean synaptic size, after which the total AZ area can no longer be maintained (DeKosky & Scheff 1990). Taken together, these findings suggest that the synaptic enlargement observed in aged hApoE4 mice is unlikely to compensate fully for the synaptic loss associated with aging.

The possible reasons and molecular mechanisms for this pattern of synaptic changes will be fully discussed later in the general discussion ( Chapter 4). However, two last points should be discussed in the context of this longitudinal study.

1) As an alternative to the hypothesis that ApoE4 is a deficient or deleterious isoform (see Chapter 4 General discussion), it may be that its level of expression declines during aging whereas that of other isoforms does not, and that the lack of ApoE results in reduced synaptic numbers in aged animals. The level of expression of ApoE in these human ApoE transgenic mice has not been characterised and it is not known how ApoE expression varies with aging, and if variation with age depends on ApoE genotype. It has been established that ApoE expression increases with aging in the brain of C57BL/6 mice (Masliah et al. 1996). It is unclear how ApoE genotype affects ApoE expression in brain cells and CSF of AD patients, with authors reporting a reduction (Beffert et al. 1999, Bertrand et al. 1995, Blennow et al. 1996, Lehtimäki et al. 1995, Yamada et al. 1995) or no change (Han et al. 1994b, Han et al.

1994a, Hesse et al. 1999, Lefranc et al. 1996) in  $\epsilon 4$  carriers compared to non- $\epsilon 4$  carriers. This emphasises the necessity to determine ApoE expression levels during aging in mice carrying the 3 ApoE isoforms to interpret precisely these synaptic changes.

2) A decrease in the Syn/nrn ratio observed in hApoE4 mice can be interpreted in 2 main ways: a decrease in synapse density or an increase in neuron density (or a combination of both) during aging. Additional measurements of the thickness of the granule cell layer suggested that tissue from aged hApoE4 mice shrinks more (either *in vivo* or during processing) than tissue from adult and young mice. The increase in neuron density was therefore the result of shrinkage rather than increase in actual neuronal numbers. Consequently, the decrease in synapse per neuron ratio observed in these mice is the result of synaptic loss.



### **3.6 ApoE polymorphism differentially influences synaptic morphometry in the MML of the DG.**

The same synaptic parameters were compared regarding the ApoE genotype for mice of the same age.

#### **3.6.1 Results**

##### **3.6.1.1 *Young mice***

In an initial study, synapse densities were estimated in the MML of the DG and in the CA1 region of the hippocampus (in the middle part of basal and apical dendrites of CA1 neurons) for WT and ApoE KO mice. This study was initiated by Masliah's report (Masliah et al. 1995) of general synaptic abnormalities in the frontal cortex and hippocampus of young ApoE KO mice.

However, for the 3 regions and for both shaft and synapse densities, no significant difference were noted between ApoE KO and WT mice (Figure 3.11). These results were published as part of a comprehensive study of ApoE KO mice in Anderson et al (Anderson et al. 1998).

When human transgenic mice were later perfused, these data were also compared to those from hApoE2 and hApoE4 mice in the DG. hApoE4 mice appeared to have the highest total and spine Syn/nn ratios but the smallest Mean AZ/syn synapse compared to the other groups (Figure 3.12). Total and spine Syn/nn ratios of hApoE4 mice were 46% and 40% greater respectively, than those of ApoE KO mice. However, Mean AZ/syn was 28% and 22% smaller in hApoE4 mice compared to hApoE2 and WT mice respectively. The same parameter was 18% smaller in ApoE KO mice compared to hApoE2 mice.

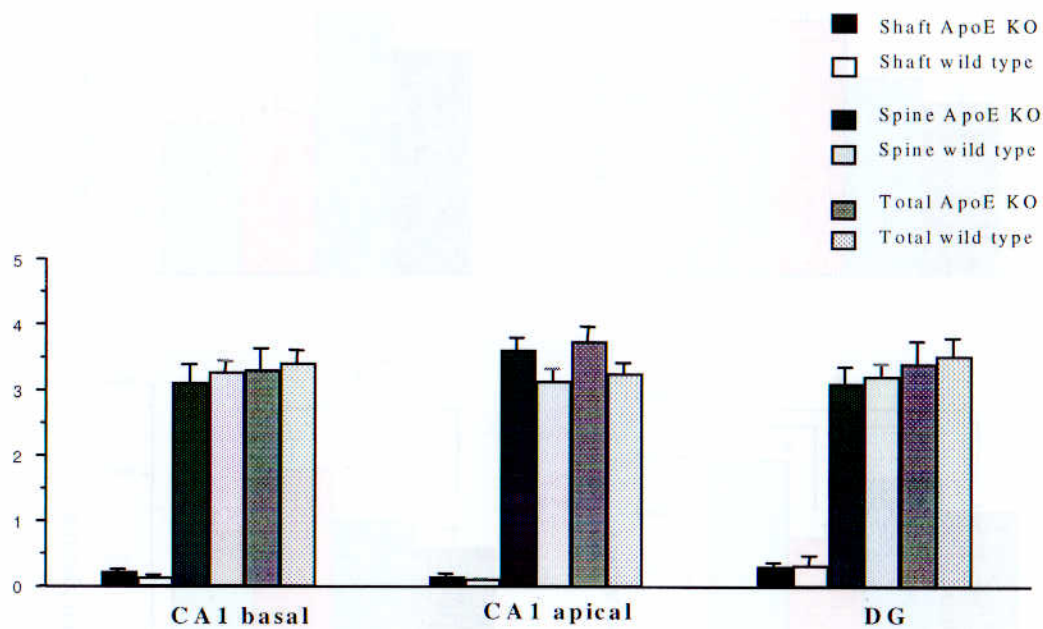


Figure 3.11 Synapse densities (expressed in synapses per  $\mu\text{m}^3$ ) estimated in the MML of DG and the middle part of basal and apical dendrites of CA1 neurons of young WT ( $n=6$ ) and ApoE KO mice ( $n=6$ ). Data are mean  $\pm$  SEM.

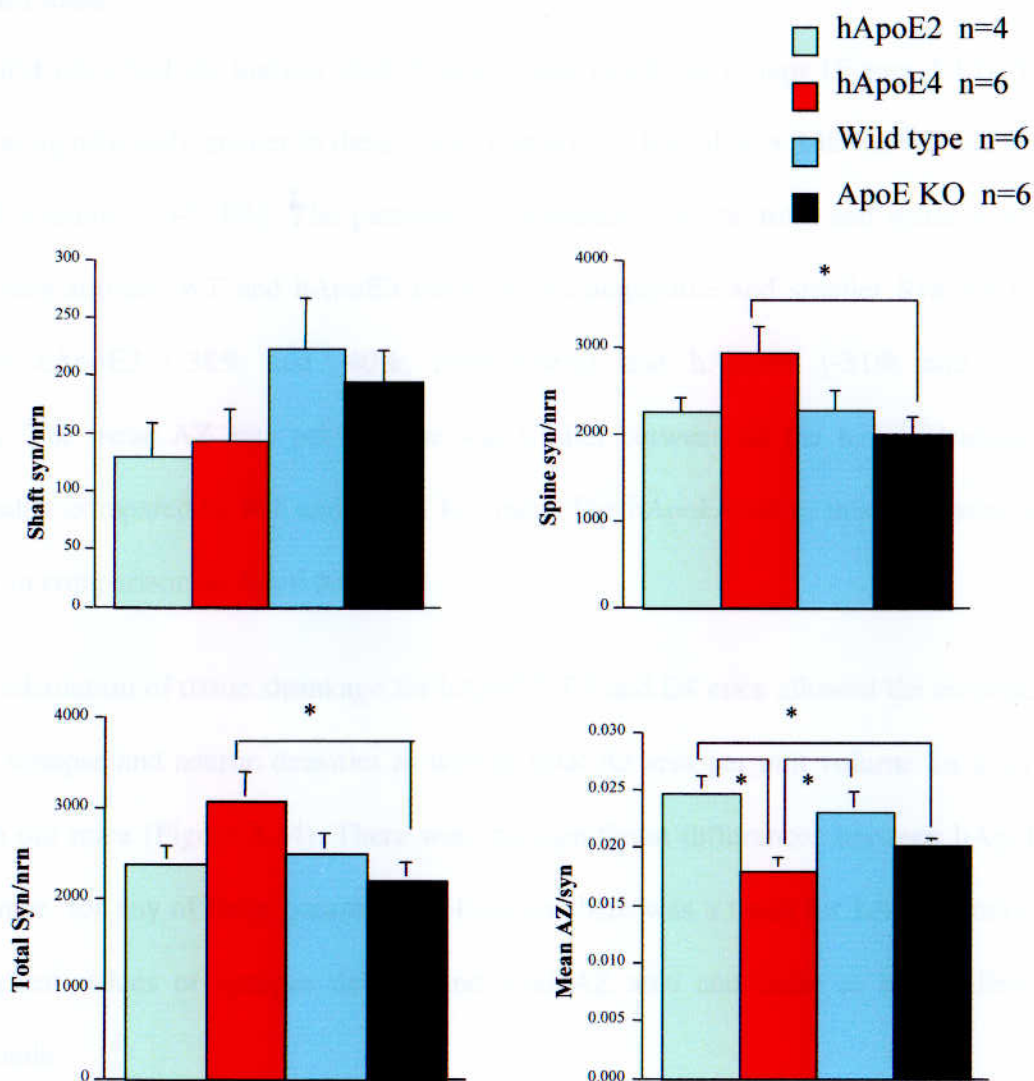


Figure 3.12 Influence of ApoE polymorphism on synaptic parameters in young mice. Mean AZ/syn is expressed as  $\mu\text{m}^2$  per synapse. Data are mean  $\pm$  SEM. Statistically significant differences are indicated by asterisks ( $p < 0.05$ ).

### **3.6.1.2 Adult mice**

hApoE4 mice had the highest shaft Syn/nrn ratio of all the groups (Figure 3.13). This parameter was significantly greater in these mice compared to hApoE3 (+ 140%), WT (+191%) and ApoE KO animals (+153%). The patterns of differences for the total and spine Syn/nrn ratios were very similar. WT and hApoE3 mice had a comparable and smaller Syn/nrn ratio compared to hApoE2 (-38% and -40%, respectively) and hApoE4 (-31% and -33%, respectively). The mean AZ area per synapse was similar between all the human transgenic mice but smaller compared to WT and ApoE KO mice. For hApoE4 mice, this parameter was 20% smaller in comparison to ApoE KO mice.

The calculation of tissue shrinkage for hApoE2, E3 and E4 mice allowed the estimation of corrected synapse and neuron densities as well as total Az area per unit volume for a set of 15-19 month old mice (Figure 3.14). There were no significant differences between hApoE2, E3 and E4 mice, for any of these parameters. However there was a trend for hApoE2 mice to have the highest values of synapse density and total AZ area and these to be smallest in hApoE3 animals.

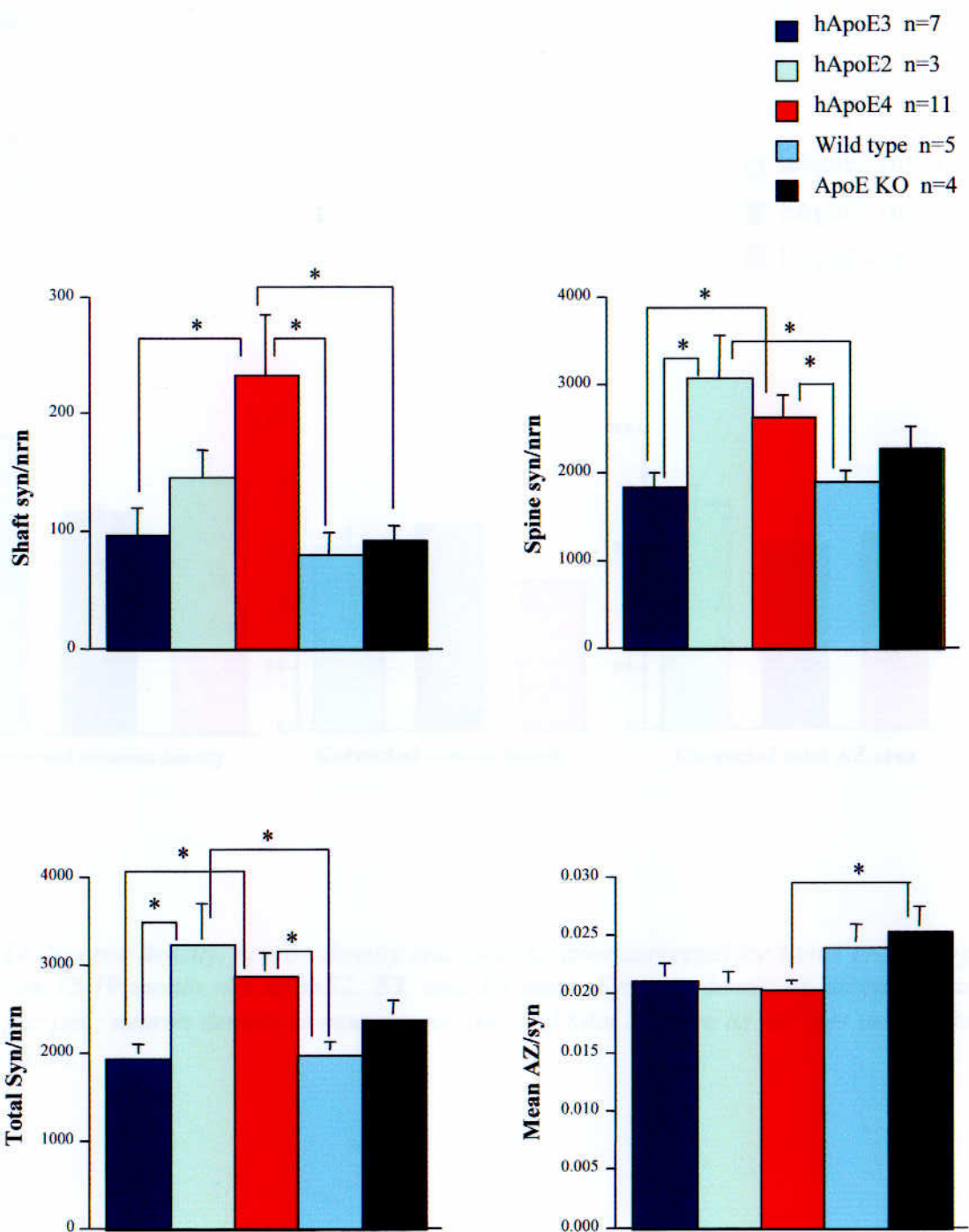


Figure 3.13 Influence of ApoE polymorphism on synaptic parameters in adult mice. Mean AZ/syn is expressed as  $\mu\text{m}^2$  per synapse. Data are mean  $\pm$  SEM. Statistically significant differences are indicated by asterisks ( $p < 0.05$ ).

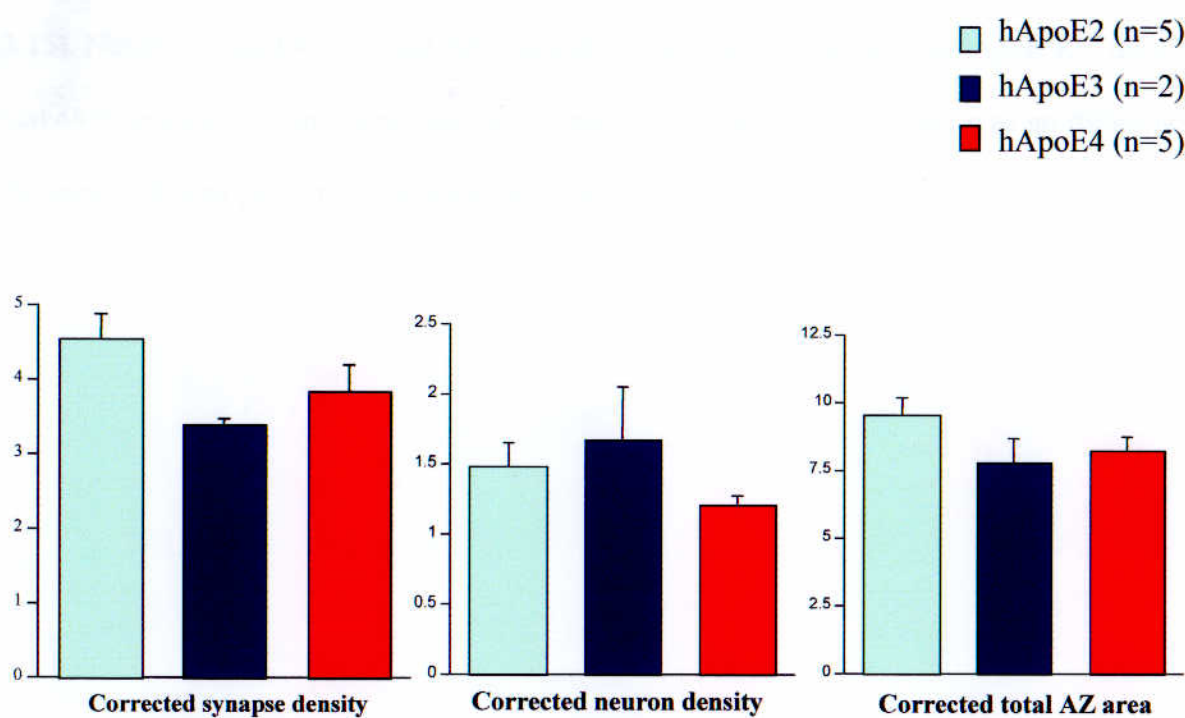


Figure 3.14 Synapse density, neuron density and total AZ area corrected for tissue processing shrinkage in 15-19 month old hApoE2, E3, and E4 mice. Synapse density is expressed as synapses per  $\mu\text{m}^3$ ; neuron density as neurons per  $\mu\text{m}^3$  and total AZ area as  $\mu\text{m}^2$  per  $\mu\text{m}^3$ . Data are mean $\pm$ SEM.

### **3.6.1.3 Aged mice**

The Syn/nrn ratio in hApoE4 mice decreased such during aging that it was 70% and 27% smaller than in hApoE2 animals, for shaft and total spine synapses respectively (Figure 3.15). Notably, hApoE4 mice had the smallest Syn/nrn ratios of all groups. ApoE KO mice also had 48% less shaft synapses per neuron compared to hApoE2 mice. There was no difference in the mean AZ area per synapse among the various groups.

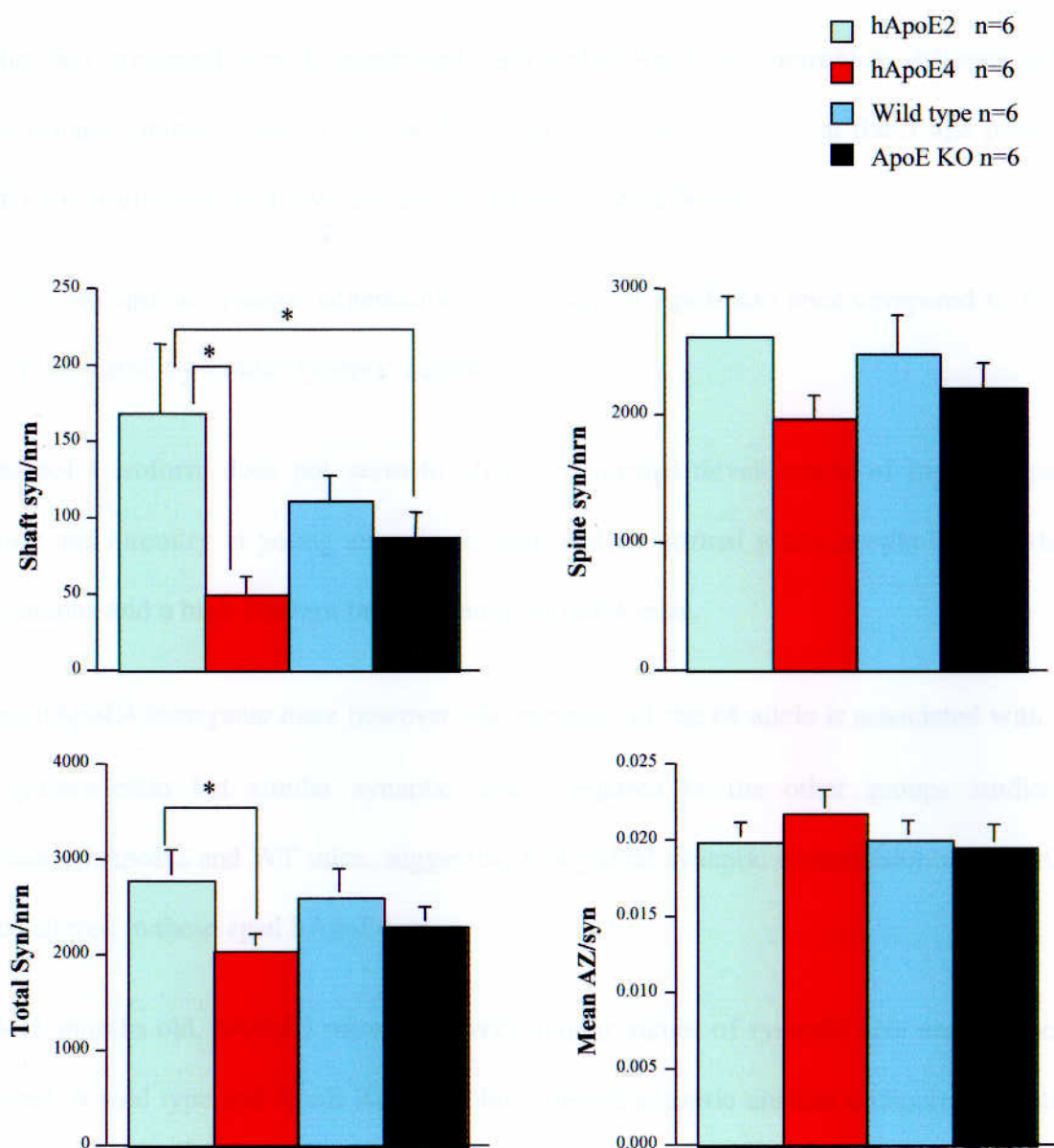


Figure 3.15 Influence of ApoE polymorphism on synaptic parameters in aged mice. Data are mean  $\pm$  SEM. Mean AZ/syn is expressed as  $\mu\text{m}^2$  per synapse. Statistically significant differences are indicated by asterisks ( $p < 0.05$ ).



### 3.6.2 Discussion

The data presented here demonstrated clearly that ApoE polymorphism differentially influences synaptic number and size in the DG of ApoE transgenic mice at the 3 age points. Four main conclusions can be drawn and are discussed in detail below:

- 1) There was no sign of synaptic degeneration at any age in ApoE KO mice compared to WT mice, as indicated by similar synaptic number and size.
- 2) The hApoE4 isoform does not seem to affect the normal development of hippocampal structure and circuitry in young animals, as indicated by normal gross morphology of the hippocampus and a high Syn/nrn ratio in young hApoE4 mice.
- 3) In aged hApoE4 transgenic mice however, the presence of the  $\epsilon 4$  allele is associated with a low Syn/nrn ratio but similar synaptic area compared to the other groups studied, particularly hApoE2 and WT mice, suggesting that global synaptic transmission in the DG may be altered in these aged hApoE4 mice.
- 4) At 14-18 months old, hApoE3 mice show very similar values of synaptic size and number compared to wild type and ApoE KO mice, but reduced synaptic number compared to both hApoE2 and hApoE4 aged-matched mice.

As previously indicated in section 3.5.1.2, the pattern of synaptic plasticity during aging in ApoE KO mice is not different from that of WT mice. In addition, values for synapse number and size are similar for both groups at all ages up to 24 months. Therefore, there is no ambiguity in the finding that the ApoE KO mice here do not develop neurodegeneration in the DG. This result contrasts with other reports describing synaptic loss in other lines of ApoE KO mice (Buttini et al. 1999, Masliah et al. 1995, Masliah et al. 1997, Masliah et al. 1999, Veinbergs et al. 1999, Veinbergs & Masliah 1999) but is in agreement with researchers who

failed to detect such a deficit (Anderson et al. 1998, Gandy et al. 1995, Parker et al. 1996, Fagan et al. 1998). Possible explanations for these discrepancies were previously mentioned in the discussion of section 3.5.1.2.

A new finding from this section is the fact that young hApoE4 mice had the highest total Syn/nrn ratio but the lowest mean synaptic area compared to hApoE2, WT and ApoE KO mice. Other groups have reported decreased labelling of synaptophysin positive presynaptic terminals and MAP2 positive neuronal dendrites in the hippocampus of identical (Veinbergs et al. 1999) and different (Buttini et al. 1999) lines of hApoE4 transgenic mice compared to hApoE3 and WT mice, as soon as 7 months of age. They reported no difference between hApoE4 mice and ApoE KO groups. Some of these results can be reconciled with the data presented here. The low shaft Syn/nrn ratio (shaft synapses are localised on dendrites) is consistent with reduced MAP2 dendritic labelling in hApoE4 mice. In addition, since the high Syn/nrn ratio in hApoE4 mice is accompanied by a small synaptic size, synaptophysin labelling of presynaptic terminals may be globally reduced, as suggested by immunocytochemical data (Buttini et al. 1999, Veinbergs et al. 1999). Electron microscopy analysis here clearly adds precision to the description of synaptic structural status compared to light microscopy immunocytochemical experiments, and suggests that decreased immunolabelling of presynaptic terminals can not necessarily be interpreted as a loss of synapses. However, in the present study, young ApoE KO mice had significantly fewer synapses than hApoE4 animals and similar synaptic size. This is in contradiction with the absence of difference in dendritic and synaptic immunolabelling between ApoE KO and hApoE4 mice reported by Buttini et al (Buttini et al. 1999) and Veinbergs et al (Veinbergs et al. 1999). A mixture of genetic backgrounds may confound the effect of the transgene (Gerlai 1996b) and ideally human transgenic mice should be compared with ApoE KO littermates. In practice, this requirement is difficult to fulfil and in the present study, it was not possible to use littermates. Nevertheless,

human transgenic mice were compared to a line of ApoE KO mice from which they were produced. In contrast, Buttini et al mixed 2 different ApoE KO lines in their ApoE KO group. Veinbergs et al, who included the same human transgenic mice used in this thesis, does not specify the origin of the ApoE KO line. However, the neurodegeneration they described in ApoE KO animals is in contradiction with data provided in this thesis from Maeda ApoE KO mice, and suggests that they might have used a different line of ApoE KO animals than the one used for production of the human transgenic mice.

In aged hApoE4 mice, the Syn/nrn ratio was the lowest compared to hApoE2, WT and ApoE KO age-matched mice. In contrast, all these groups did not differ in terms of synaptic size. These data are difficult to compare with the literature because, as yet, no study has tried to assess the influence of the ApoE2 isoform on synaptic integrity. Often, groups in human studies are designed as  $\epsilon$ 4 positive and  $\epsilon$ 4 negative, and in transgenic mice models only the ApoE3 isoform is compared to the ApoE4 one. Although studies of human ApoE transgenic mice have not been carried out beyond 12 months of age (Buttini et al. 1999, Veinbergs et al. 1999), their results indicate that synaptic integrity in the hippocampus and the frontoparietal cortex is affected in hApoE4 mice compared to hApoE3 and WT mice. There were no aged hApoE3 mice available for the present study, however, in agreement with the 2 other transgenic studies mentioned above, a difference in synaptic number could be seen between aged hApoE4 and WT mice. In AD patients, those carrying the  $\epsilon$ 4 allele display less synaptophysin immunoreactivity in the frontal cortex than those without the  $\epsilon$ 4 allele (Miller et al. 1994, Masliah et al. 1996). Heinonen (Heinonen et al. 1995a) observed a similar trend in the hippocampus and the EC of AD patients but differences were not significant. In contrast, Blennow (Blennow et al. 1996) found no difference in the level of Rab3 immunoreactivity in the IML of the DG associated with ApoE genotype. As yet, no study has examined the effect of ApoE polymorphism on synaptic integrity in non-demented elderly. Since the  $\epsilon$ 2 allele may

have a protective role against AD (Corder et al. 1994, Lippa et al. 1997), it is intriguing that aged hApoE2 transgenic mice have the highest number of synapses and that, consistently, mice carrying the  $\epsilon 4$  allele associated with a high risk factor of AD (Saunders et al. 1993a), have the lowest number (27% fewer synapses than the hApoE2 mice). Such an effect and its possible functional consequences will be discussed in detail in the general discussion ( Chapter 4).

Two major points that may have influenced these data, showing marked differences between hApoE2 and hApoE4 transgenic mice, should be discussed. First, 2 different investigators estimated synapse densities in both lines. This could have potentially introduced some bias and produced false positive or negative significant results. However, both investigators carefully agreed on criteria to count synapses, and checked that their counts were matching for 2 animals. Therefore, bias introduced by investigators is unlikely. Secondly, both transgenic lines express a different number of copies for the ApoE gene. hApoE2 mice possess 8 copies whereas hApoE4 mice have 2, with more ApoE being produced in hApoE2 mice than in hApoE4 mice (Xu et al. 1995). It is arguable that less ApoE protein available to cells may be associated with a failure to maintain synapse number, and affect age-related synaptic changes, in comparison with lines that produce more ApoE, and irrespectively of the isoform. Although such a quantitative effect cannot be ruled out, there are indications from this study and from the literature that the identity of the isoform is crucially linked to the effects observed under experimental conditions. In favour of this argument, this study showed that only hApoE4 mice are unable to maintain synapse numbers during aging. In contrast, ApoE KO mice, which express no ApoE, do not exhibit such age-related changes suggesting that the level of ApoE expression is not an issue.

At 14-18 months old, hApoE3 mice showed very similar values of synaptic size and number compared to those of WT and ApoE KO mice, but reduced synaptic number compared

to both hApoE2 and hApoE4 aged-matched mice. From the results of Buttini and Veinbergs' work (Buttini et al. 1999, Veinbergs et al. 1999), a better synaptic integrity was expected in hApoE3 compared to hApoE4 mice. However, the data presented in section 3.5.1.4 showed that the synapse per neuron ratio in hApoE4 mice continues to decline beyond 14-18 months of age, suggesting that a difference between hApoE3 and hApoE4 mice may emerge later in life. The hypothesised differential capacity for maintenance of synaptic integrity of the 3 ApoE isoforms may be revealed at an older age, when synaptic and neuronal injuries are more frequent. It is also important to mention that although size and number of synapses are good indicators of synaptic wiring, they do not indicate the degree of efficacy of synaptic transmission. It has been suggested that a number of synaptic proteins (neurotransmitter receptors, synaptic vesicles proteins, for example) might be up-regulated to compensate synaptic loss and maintain synaptic transmission (Barnes 1994, Barnes 1999). Electrophysiological investigations in the PP of human ApoE transgenic mice would help determine if the efficacy of synaptic transmission differs between hApoE3 and hApoE4 transgenic mice.

### **3.7 Note on the influence of gender on synaptic plasticity**

Sexual differentiation in the brain occurs early in life, during early prenatal or postnatal development. The hippocampus is one region to exhibit sexually dimorphic features of its structure and function (Desmond & Levy 1997). With regard to the structure of DG, males of some strains of mice with large numbers of granule cells have more granule neurons than females (Tanapat et al. 1999, Wimer & Wimer 1985). In rats, males have more mossy fibres synapses in the hilus (Parducz & Garciassegura 1993) whereas females have more mossy fibres synapses in the CA3 region (Madeira et al. 1991). Several studies have also demonstrated the influence of ovarian steroid hormones on hippocampal structure and function in adulthood. In the CA1 region, dendritic spine and synapse densities correlate positively with estrogen levels, after experimental manipulations and in response to natural fluctuations in females (Desmond & Levy 1997, McEwen et al. 1997, Woolley et al. 1990, Woolley & McEwen 1992). Similarly, in adult female rats, granule cell proliferation correlates with circulating estrogen levels (Tanapat et al. 1999).

Although this is still controversial, women generally show a higher rate of incidence of AD compared to men (Bickeboller et al. 1997, Farrer et al. 1997, Poirier et al. 1993b). Whilst this observation has no clear basis, the fact that estrogen replacement therapy lowers the risk of AD in women (Kawas et al. 1997, Tang et al. 1996) suggested that the loss of estrogens at menopause may be crucial. Interactions between estrogen changes and AD pathology are not well known. A recent study showed that estradiol inhibits A $\beta$  peptide secretion in cultured neurons (Xu et al. 1998a), supporting the idea that estrogen loss may contribute to AD pathology.

A direct comparison of  $\epsilon 4$  heterozygous men and women revealed a significant 2 fold increase of AD risk in women (Payami et al. 1995, Payami et al. 1996 but see Corder et al. 1995b). It is of particular interest that ApoE expression is regulated by estradiol in astrocytes and microglia in female rats (Stone et al. 1997, Stone et al. 1998). Namely, ApoE mRNA levels increase at proestrus concomitantly with high concentrations of circulating estradiol and intense synaptogenesis in the CA1 region of the hippocampus and in the arcuate nucleus of the hypothalamus (Stone et al. 1997).

Taken together, these findings suggest that both gender and ApoE polymorphism may influence the synaptic plasticity associated with aging in the hippocampus of ApoE transgenic mice. The groups of animals used in this thesis were composed of males and females, with a large male dominance. It would have been interesting to segregate males and females into groups of equal size to explore both the effects of ApoE genotype and gender on synaptic plasticity associated with aging. Since animal numbers were reduced, it was necessary to group both sexes together for the general analysis (sections 3.5 and 3.6). However, for interest, this section of the thesis analyses trends associated with gender on a subset of mice, and confirms that the results obtained in aged animals are still valid for only-male groups.

### **3.7.1 Results**

For this analysis, a subset of hApoE2, E3 and E4 mice were used. Age was the criteria to choose the animals. Since females are menopausal at approximately the age of 12-14 months (Silver 1995), all the animals under 14 months of age were excluded. This ruled out the possible influence of hormonal variations (which could not be monitored) on structural synaptic and neuronal plasticity in the DG of females.

Two-way ANCOVA (using age as a covariate) showed that both ApoE genotype and gender had a significant effect ( $p=0.003$  and  $p=0.001$  respectively) on Syn/nrn ratio, but that

there was no interaction between genotype and gender (p=0.438). ApoE genotype and gender had no significant effect on mean AZ area per synapse (p=0.4 and p=0.23 respectively).

Syn/nrn ratio and mean AZ/syn were subsequently compared between females and males for individual transgenic line. The results of the statistical analysis are presented in Table 9.

Line	Gender	Syn/nrn	Mean AZ area per syn	n	Age
hApoE2		<b><i>p=0.005*</i></b>	<b><i>p=0.503</i></b>		
	female	2757	0.0207	2	14
	male	2963	0.0198	7	19
hApoE3		<b><i>p=0.079</i></b>	<b><i>p=0.83</i></b>		
	female	1729	0.021	4	14
	male	2189	0.020	3	17.3
hApoE4		<b><i>p=0.046*</i></b>	<b><i>p=0.26</i></b>		
	female	2353	0.0188	4	15
	male	2642	0.0215	13	17.6

Table 9 Effect of gender on synaptic morphometry in the DG of ApoE transgenic mice. Data presented are means. Mean AZ per synapse is expressed in  $\mu\text{m}^2$  per synapse, age in months. The effect of gender was tested by ANCOVA with age as a covariate (p value in bold, with an asterisk when <0.05).

Gender did not affect the mean AZ area per synapse. In contrast, it had a significant effect on the Syn/nrn ratio for hApoE2 and hApoE4 mice but not for hApoE3 mice. In general, females had a smaller Syn/nrn ratio than males, in the 3 groups of ApoE transgenic mice.

Sections 3.5 and 3.6 showed that 1) the synapse per neuron ratio decreases significantly with age only in hApoE4 mice, and 2) that aged hApoE4 mice had a smaller Syn/nrn ratio compared to hApoE2. Since groups used for this analysis were mixed in gender (and had different ratios of male/female), results may have been distorted by the presence of females.



Therefore, the comparison of adult and aged hApoE4 mice was reanalysed with the exclusion of females. The previous result was reinforced: aged hApoE4 males had a 36 % smaller synapse per neuron ratio compared to adult hApoE4 males ( $p=0.01$ ), instead of the 29% difference observed between groups of mixed gender ( $p=0.04$ ). The changes in mean AZ area were mildly altered (+7% for mixed groups and + 2.5% for only-male groups). It was not possible to remove females from the adult hApoE2 group because of its small size (1 male/2females). The difference in Syn/nrn ratio between aged hApoE2 and hApoE4 could not be affected by gender as both these groups were only composed of males.

### **3.7.2 Discussion**

Although this analysis was performed on groups of small size, it revealed an interesting trend. One common observation was a lower Syn/nrn ratio in females of all ApoE transgenic groups compared to males. Whilst this might suggest reduced synaptic connectivity in females, such conclusions would need confirmation with further study. As mentioned previously, a reduced Syn/nrn ratio may, for example, be compensated by pre and/or postsynaptic changes to maintain the same level of synaptic transmission (Barnes 1994, Barnes 1999). Since there was no difference in the average synaptic size, small synaptic number did not seem to be compensated for by increased synapse size. This may reflect an absence of reactive synaptic plasticity in menopausal females against age-related synaptic loss. This hypothesis is supported by findings of a reduction of sprouting after ovariectomy and its reinstatement by estrogen replacement (Morse et al. 1986, Morse et al. 1992). Moreover, estrogen also regulates granule cells proliferation in the DG of female rats (Tanapat et al. 1999). It is not known how neurogenesis of granule cells is affected by menopause, but ovariectomy clearly reduces granule cells proliferation. Reduced number of granule cells, but more importantly reduction of postsynaptic sites located on their dendritic tree in the MML, may explain why female mice at menopause have a smaller Syn/nrn ratio than males in this study.

There are clear indications that estrogen –induced sprouting after ECL operates via an ApoE-dependent mechanism. ECL is used as a model of the deafferentation occurring in AD. Both in AD and ECL, the loss of PP afferents is followed by compensatory sprouting in the ML of DG. Most ApoE KO mice are deficient for this sprouting response (Anderson et al. 1998, Fagan et al. 1998, Krzywkowski et al. 1999, Masliah et al. 1996, Teter et al. 1999a). Whereas estrogen replacement restores synaptic sprouting in the ML of DG in ovariectomized WT mice, it does not in ovariectomized ApoE KO mice (Stone et al. 1998, Teter et al. 1999a). This suggests that estrogen stimulates sprouting through up-regulation of ApoE expression (Stone et al. 1997).

A recent study using hippocampal slices in culture taken from ApoE KO mice showed that ApoE3 added to culture medium restores sprouting whereas ApoE4 is only half as efficient (Teter et al. 1999b). Unfortunately, the results presented here are based on very small samples and cannot reasonably be used for comparison of females with different ApoE genotype to identify a possible interaction of gender with ApoE polymorphism. Other reports from human AD patients and ApoE transgenic models have shown respectively reduced neuronal remodelling and reduced GAP43 immunoreactivity in various brain regions of  $\epsilon$ 4 individuals (Arendt et al. 1997, Veinbergs et al. 1999), suggesting that presence of the  $\epsilon$ 4 allele may be associated with impaired sprouting. The combined effect of an estrogen deficient state with the influence of the  $\epsilon$ 4 allele on reactive synaptic plasticity and neuronal repair may account for the increased risk of AD in  $\epsilon$ 4 females.

### **3.8 Influence of ApoE polymorphism on glutamate immunolabelling in the neuropil of the MML.**

Glutamate is an amino acid with multiple biological roles and it is almost omnipresent throughout the hippocampus. As well as being part of a metabolic pool in all cells, it is also used as a neurotransmitter in various populations of neurons. Therefore in both cases, glutamate is essential to the maintenance of neuronal activity.

In neurons, glutamate is present in different compartments and cellular regions where it plays various roles. In synaptic terminals, the amino acid is contained in synaptic vesicles, ready for extracellular release. Presynaptic depolarisation triggers a cascade of events that leads to the release of glutamate in the synaptic cleft which then binds to a variety of postsynaptic receptors to mediate its effects. The excess of glutamate present in the extracellular space is removed rapidly by an uptake transporter into glial cells, where it is converted into glutamine by glutamine synthetase. The adequate replenishment of transmitter glutamate is assured by the transport of glutamine from glial cells to the nerve terminals where glutamine is converted into glutamate by glutaminase (reviewed in (Erecinska & Silver 1990)). Immunocytochemical studies have also located the amino acid in mitochondria and in dendrites. Transport of glutamate from the cytosol into mitochondria delivers the substrate to glutamate dehydrogenase to provide reducing equivalents for mitochondrial respiration. The reason for the presence of glutamate in dendrites is less clear. Since a glutamate transporter has been localised at the postsynaptic membrane (Rothstein et al. 1994), glutamate may be uptaken into spines and dendrites to enter the glutamine-glutamate cycle. Another intriguing hypothesis is the use of glutamate for local dendritic protein synthesis. An increasing number of dendritic mRNAs have been discovered, and the presence of translational machinery in dendrites of hippocampal

neurons in culture has been established (Tiedge & Brosius 1996) supporting the current view that local protein synthesis in dendritic domains serves the management of dendritic protein repertoires on site. Additionally, glutamate is involved in many other biochemical pathways, notably as a precursor for the inhibiting neurotransmitter GABA (reviewed in (Erecinska & Silver 1990)).

In AD, glutamatergic neurotransmission is severely compromised. Glutamatergic neurons and terminals degenerate in the EC and the hippocampus (Braak & Braak 1991, Hyman et al. 1984, Pearson et al. 1985, Procter et al. 1988). The glutamatergic PP arising from layer II of the EC and terminating on the dendrites and spines of granules cells in the DG is particularly affected (Amaral 1995, Hyman et al. 1984). Up to 90% of its terminals are lost (Honer et al. 1992b, Sze et al. 1997) and there is an 83% decrease in glutamate level in AD patients compared to aged controls (Hyman et al. 1987b). The status of glutamate receptors in the pathology is unclear. Generally, most classes of receptors seem to decrease (Aronica et al. 1998, Cowburn et al. 1988).

Since possession of the  $\epsilon 4$  allele is a risk factor for AD (Corder et al. 1993) and the synaptic connectivity in the MML of the DG is significantly affected in this pathology (Heinonen et al. 1995b, Masliah et al. 1994b), ApoE polymorphism may influence glutamate concentrations in the DG. As shown in section 3.6.1.3, aged hApoE4 transgenic mice have a reduced number of synapses per neuron compared to age-matched hApoE2 mice. In the present part of this study, relative glutamate concentrations were estimated by immunogold labelling in the MML of the DG of aged hApoE2, E3 and E4 transgenic mice to examine a possible influence of ApoE polymorphism on glutamatergic concentrations in presynaptic boutons and dendrites, since these 2 sites play an important role in neuronal activity and memory formation.

### **3.8.1 Results**

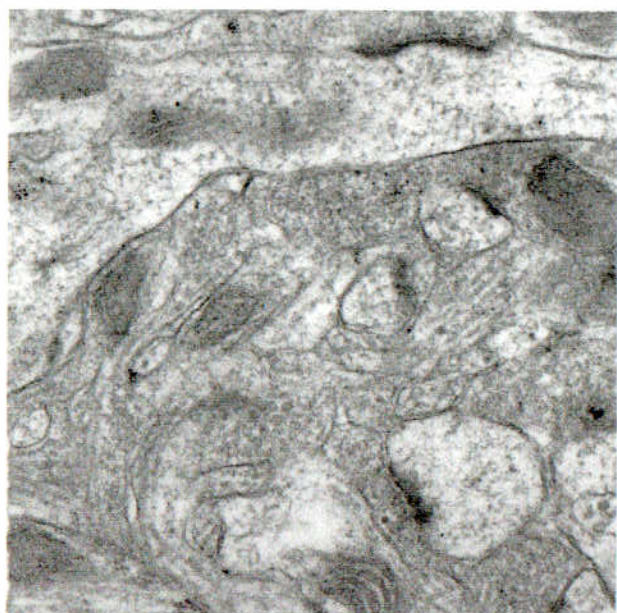
For the immunocytochemistry experiment, each transgenic group initially included 6 mice. One mouse from the ApoE3 group was removed from the final analysis because of the absence of immunogold labelling which was attributed to inadequate block polymerisation.

#### **3.8.1.1 Immunolabelling specificity**

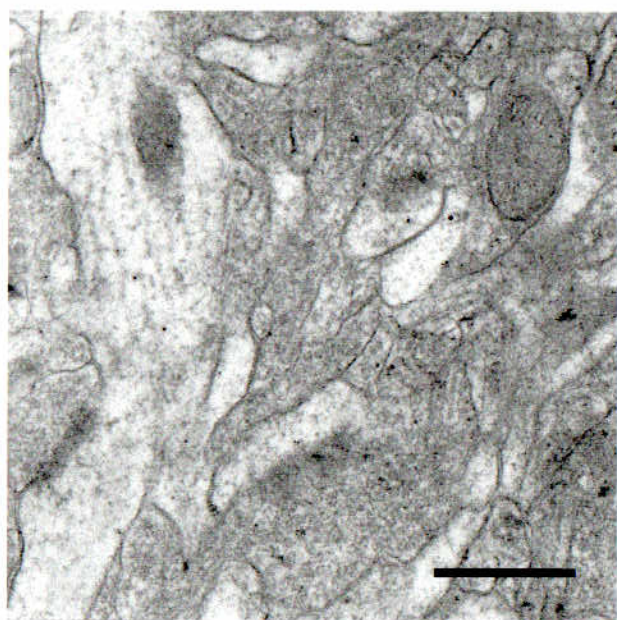
Very low particle densities were found on control sections treated with no primary antibody or with normal rabbit serum (Figure 3.16) in all transgenic groups. Virtually no gold particles were seen on resin devoid of tissue such as the lumen of blood vessels, confirming that with the chosen experimental conditions, there was no background labelling. Therefore, immunolabelling quantification in the structures of interest (see 3.8.1.3) did not require background subtraction.

#### **3.8.1.2 Qualitative distribution of glutamate immunogold labelling**

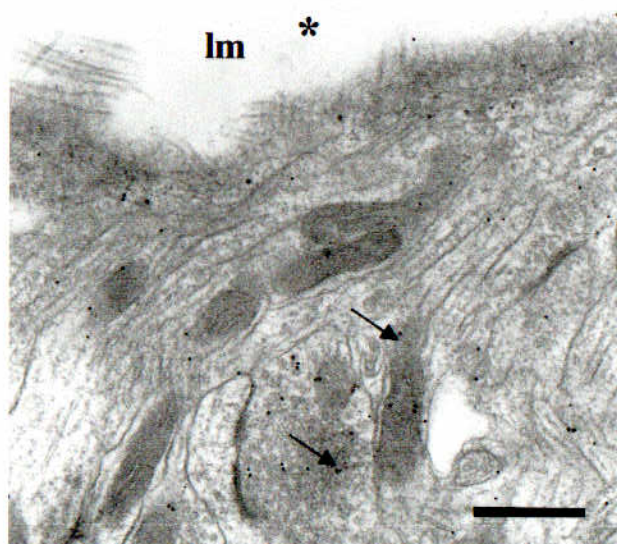
In the MML, gold particles were found over neuronal elements (dendrites and presynaptic boutons) and glia (Figure 3.17). This distribution did not appear to vary between the 3 ApoE transgenic groups. Presynaptic boutons displayed the highest density of labelling. Most labelled synapses were asymmetrical and some postsynaptic spines displayed gold particles. Even in mice with high immunogold labelling, not all presynaptic boutons were labelled. Occasionally, gold particles appeared on mitochondria contained in synaptic terminals and in dendritic profiles. In dendrites, immunogold labelling was scattered and generally much lower than in presynaptic boutons. Glial processes in the neuropil were rarely seen and displayed little or no labelling.



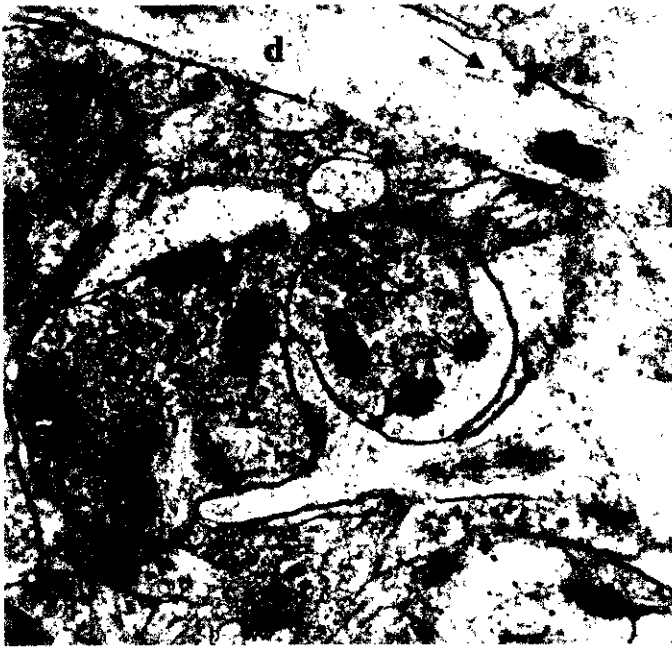
**No primary  
antibody**



**Normal rabbit  
serum**



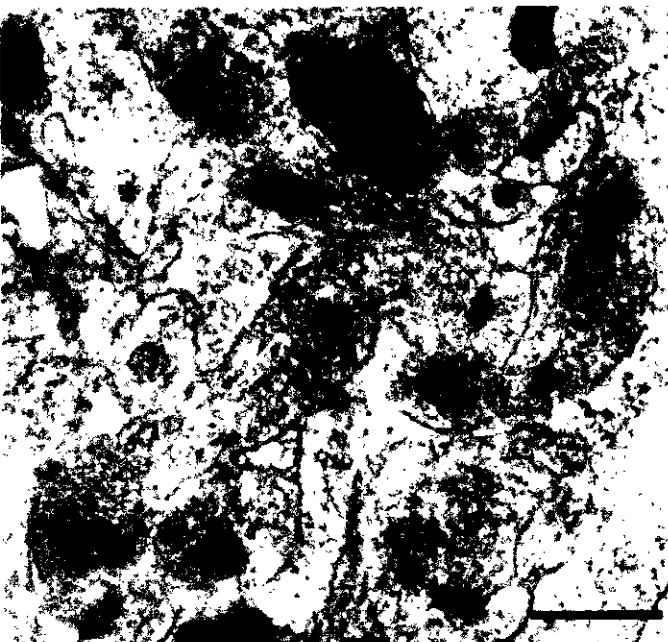
**Absence of labelling on  
tissue free-resin\*  
with anti-glutamate  
antibody**



**hApoE2**



**hApoE3**



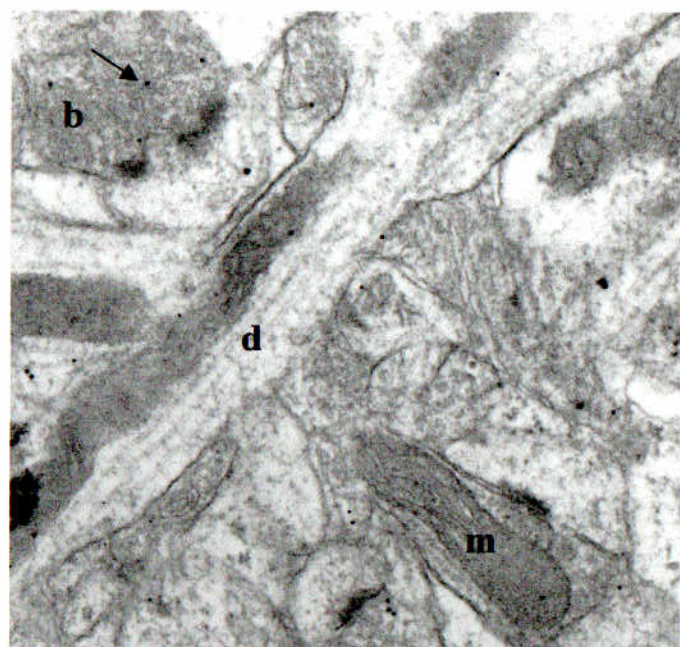
**hApoE4**

*Figure 3.16 Specificity of glutamate immunogold labelling. On both sections incubated either without anti-glutamate primary antibody, or with normal rabbit serum instead of the primary antibody, virtually no gold particle labelled the tissue. In experimental sections incubated with anti-glutamate antibody, only the neuropil and presynaptic terminals (arrows) are labelled, in contrast to tissue-empty resin such as the lumen (lm) of a blood vessel. Scale bar is 0.5µm.*

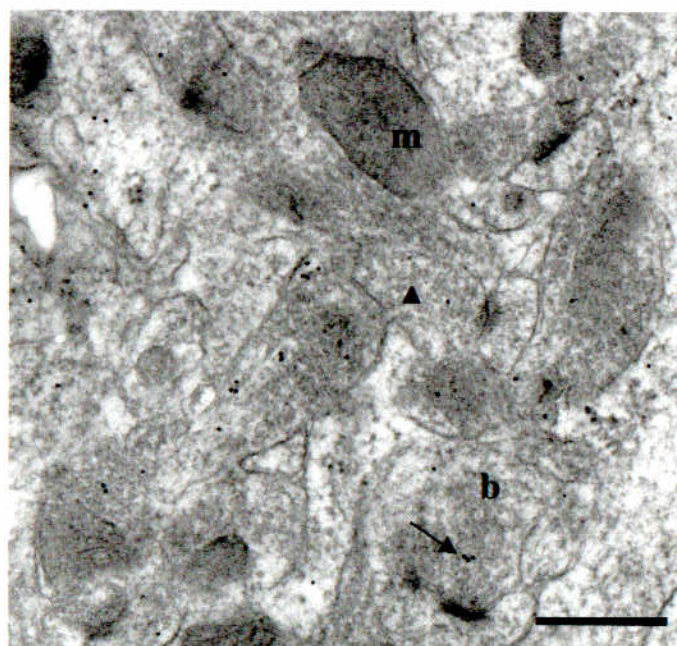




**hApoE2**



**hApoE3**



**hApoE4**

*Figure 3.17 Differential concentrations of glutamate immunogold labelling in the MML of ApoE2, E3 and E4 aged transgenic mice. Glutamate immunogold reactivity (arrows) is mainly observed in presynaptic boutons (b), associated with synaptic vesicles (arrowhead). Dendrites (d) and mitochondria (m) displayed less dense labelling. Note the greater intensity of immunolabelling in presynaptic boutons in hApoE2 mice compared to hApoE3 and E4 mice. Scale bar is 0.5µm.*

3.8.1.3 Quantitative evaluation

Although mice were chosen to be in the same age range, there was a small but significant difference in age between the transgenic groups (see Table 10).

Line	n	Age (months)
hApoE2	6	18.6±0.5†
hApoE3	5	16±0.9
hApoE4	6	19.6±0.4*

Table 10 Profiles of mice used for quantification of glutamate immunogold labelling. Mean age± S.E.M is significantly smaller in the hApoE3 group compared to hApoE2 and hApoE4, indicated respectively by † and \*.

Since glutamate concentrations, and release, may vary with age in rodents (Myhrer 1998), the 3 transgenic groups were age-matched for the following statistical analysis, by using an ANCOVA with age as a covariate.

Figure 3.18 shows the mean gold particle density over presynaptic boutons and dendritic profiles in the MML of aged hApoE2, E3 and E4 transgenic mice. The highest number of gold particles for both compartments was found in the hApoE2 group, while hApoE3 had the smallest density and hApoE4 an intermediate value. In presynaptic boutons, there was a –33% significant difference between hApoE2 and hApoE3 (p=0.023) and a –16% difference between hApoE2 and hApoE4 mice (non significant). Although a similar pattern was found in the dendritic compartment, differences were not significant. Notably, gold particle density in presynaptic boutons was about twice greater than in dendrites, for all transgenic

groups. This is consistent with the view that glutamate serves as a neurotransmitter in presynaptic boutons while it may have a metabolic function in dendrites instead.

Intriguingly, the pattern of differences of total synapse density between the 3 transgenic groups mirrored that of both bouton and dendrite gold particle densities. Synapse density for hApoE2 mice was 58% and 21% higher than that for hApoE3 ( $p=0.0009$ ) and hApoE4 ( $p=0.051$ ) respectively. The relationship between these 2 parameters was confirmed by a significant positive correlation ( $p=0.014$ ;  $r=0.58$ ). Figure 3.19 shows that when all the transgenic groups are pooled in one group, mice with small synapse density also display small gold particle density. In contrast, mice with high synapse density display high gold particle density, but there was no such correlation within individual transgenic groups. This result raised an important question: is gold particle density in presynaptic boutons more influenced by ApoE genotype or by synapse density? The previous regression analysis showed that about 34% of the variation in gold particle density is accounted for by the variation in synapse density ( $r^2=0.34$ ). To test that ApoE genotype also contributes to the immunolabelling, differences of the mean gold density between ApoE transgenic groups were tested with an ANCOVA, using synapse density as a covariate. The analysis demonstrated that a significant difference between the ApoE2 and ApoE3 groups persists, even when the variations of synapse density are removed. This confirms that there is a 'true' difference in gold particle density between ApoE transgenic groups, which is partly accounted for by ApoE genotype.

There was no significant correlation between synapse density and gold particles density in dendrites within individual transgenic groups or for the whole pool of mice. This last result was expected since these 2 measurements are made in independent compartments: dendrites present in the MML are likely to arise from cells of the granule cells layer, whereas synapses counted for synaptic density most likely arise from EC cells that project to the MML.

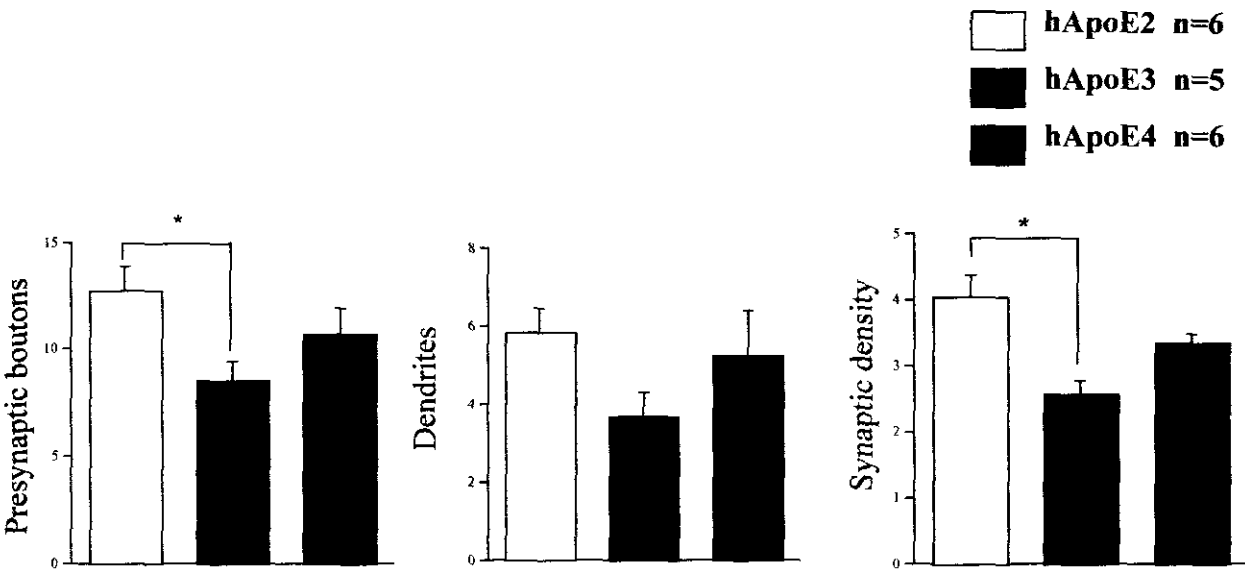


Figure 3.18 Quantification of immunogold labelling in the neuropil of the MML of the DG of human transgenic mice. Gold density is expressed as number of gold particles per  $\mu\text{m}^2$  area of presynaptic bouton or dendrite. Synaptic density is expressed as synapses per  $\mu\text{m}^3$  of tissue. Significant differences are indicated by an asterisk ( $p < 0.05$ ).

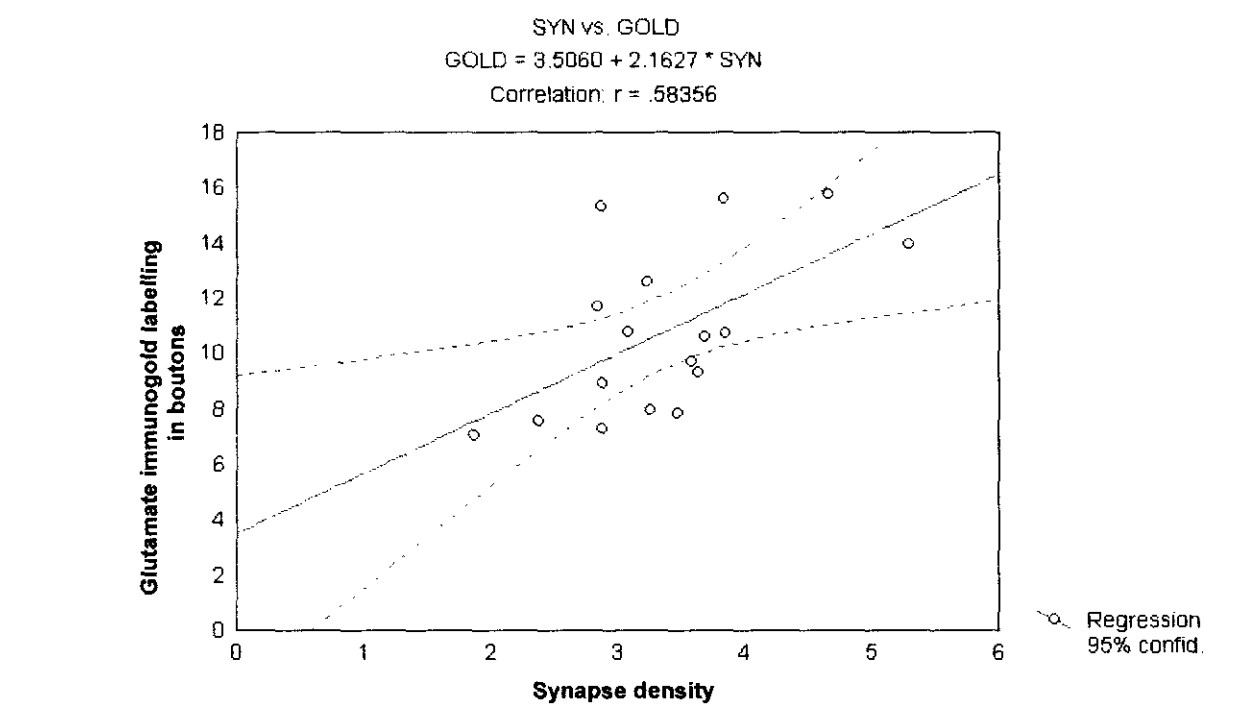


Figure 3.19 Linear regression analysis between synapse density and glutamate immunogold labelling in presynaptic boutons of the MML. Data from ApoE2, E3 and E4 mice were all included for this analysis. Density of gold particles and synapses are expressed per  $\mu\text{m}^2$  and  $\mu\text{m}^3$  respectively

### 3.8.2 Discussion

These results show that ApoE polymorphism in transgenic mice is associated with differences in glutamate immunolabelling density in presynaptic boutons and dendrites of the MML.

#### 3.8.2.1 *Technical comments*

The validity of these results is discussed and suggests strongly that the observed differences in immunolabelling reflect 'true' physiological differences between the 3 ApoE transgenic groups rather than experimental artefacts. However, the following caveats should be considered.

##### *1) Possible leakage of glutamate within and outside the cell.*

It is conceivable that glutamate from the transmitter or metabolic pool may have diffused or leaked into other cellular compartments before complete fixation of the tissue. However, all the animals were perfused rapidly intracardially and for the same amount of time (25 minutes). Although it is not known if ApoE polymorphism affects the quality or speed of tissue fixation, it seems unlikely that the hippocampus, which is a small structure (relative to the volume of fixative for intracardial perfusion) and well supplied with blood vessels, would even be affected by such differences.

##### *2) Experiment variability*

Sections from each animal and for each transgenic group were treated all simultaneously and with the same solutions, to keep the variability of the whole procedure to a minimum.

##### *3) Specificity of labelling*

Parallel control sections demonstrated that both the primary anti-glutamate antibody and the gold conjugated antibody were specific. There was virtually no background staining on these

sections or on tissue-free resin region of experimental sections (even in mice demonstrating the highest density of immunogold labelling).

#### *4) Location of image analysis*

Presynaptic glutamate immunolabelling has been shown to vary across the outer 2 thirds of the ML of the DG, with higher density in the outer ML compared to the middle ML (Bramham et al. 1990). If images for gold counting were taken outside the MML within one animal or one group, gold densities would not be comparable and bias the results. However, this bias would also introduce great variability, which was not observed in the present results.

#### *5) Quantification of immunolabelling*

Finally, the aim of this study was to compare relative immunogold particles density, not to determine absolute glutamate concentrations. Although antigen concentration generally varies linearly with gold particle density, Bramham (Bramham et al. 1990) also reported a non linear relationship between these 2 parameters. This implies that low concentrations of fixed glutamate result in a disproportionately strong labelling compared with higher concentrations. If that were the case in this study, differences between the groups would be even more pronounced.

#### **3.8.2.2 Comments on general pattern of immunogold labelling in the MML of DG.**

The pattern of anti-glutamate immunogold labelling in the DG and the hippocampus has been described extensively by several authors (Bramham et al. 1990, Grandes & Streit 1991, Gundersen et al. 1998, Hyman et al. 1987b, Liu et al. 1989, Wenzel et al. 1997). The labelling observed in the present study is in agreement with these reports.

In the MML, gold particles were observed in various compartments. The highest density was detected in presynaptic terminals, which is consistent with glutamate acting as a neurotransmitter in this brain region. The glutamatergic nature of the PP has been well

established with a variety of methods (Grandes & Streit 1991, Nadler et al. 1976, Ottersen & Storm Mathisen 1985, Ottersen 1989, Storm Mathisen et al. 1983). Since glutamate is also a precursor for the neurotransmitter GABA, some labelled symmetric synapses may be GABAergic rather than glutamatergic. In these inhibitory terminals, glutamate concentrations are then very low, probably due to the rapid conversion into GABA (Ottersen & Storm Mathisen 1984, Ottersen 1989, Ottersen & Storm Mathisen 1985). In mice with low immunogold density, the results presented here may have been lowered by the presence of numerous GABAergic terminals. However, this is unlikely because GABAergic terminals represent a very low proportion of the whole set of terminals in the MML, normally dominated with glutamatergic ones (Amaral 1995, Grandes & Streit 1991). Therefore, even in this extreme case, a high proportion of GABAergic terminals would imply that glutamatergic terminals had been lost, probably reflecting dysfunction or neurodegeneration of glutamatergic neurons.

Glutamate labelling was also detected in the dendrites present in the neuropil of the MML. Its density was about half of that in presynaptic boutons, suggesting that in dendrites, glutamate serves for metabolism rather than neurotransmission. Most dendrites from the MML are likely to belong to glutamatergic granule cells but there are also non-glutamatergic cell types (see introduction section 1.2.1) contributing to the neuropil connectivity.

In conclusion, it is reasonable to suggest that most labelled presynaptic boutons use glutamate as their neurotransmitter, whereas labelled dendrites may reflect the involvement of the amino acid for metabolic activity.



### ***3.8.2.3 Influence of ApoE genotype on glutamate immunogold labelling: possible causes and functional consequences.***

As shown in Figure 3.18, glutamate immunogold labelling varies with ApoE genotype both in presynaptic boutons and in dendritic profiles. Such results have never been reported before. The mechanism behind such effects is yet unknown.

Glutamate can be synthesised via several pathways in neurons (reviewed by (Erecinska & Silver 1990). The main source of glutamate at the presynaptic terminal site, originates from the uptake of glutamine released by glial cells, followed by its conversion into glutamate. Variable amounts of presynaptic glutamate may therefore be explained by alteration of any step in the glutamine-glutamate cycle, although, as yet, there has been no report of ApoE influence on any of these stages. For example, ApoE genotype may modify the availability or the number of excitatory amino acid transporters in glial cells or neurons. In the temporal cortex of AD patients, up to 60% of glutamate uptake sites are lost (Cowburn et al. 1988) and there is an 80% loss of glutamate concentrations in the PP terminal zone (Hyman et al. 1987b). In both cases, these changes detected by light microscopy were interpreted as a consequence of synaptic terminal loss. This explanation however does not hold for the results presented here. Glutamate concentrations were measured at the EM level, from remaining synapses and expressed per unit area of presynaptic bouton. Therefore, differences in immunogold labelling between the ApoE transgenic groups cannot be attributed to a difference in synaptic density. The positive correlation between synapse density and gold particle density suggests that in animals with a higher synaptic density, high glutamate immunogold labelling may better synaptic transmission.

A speculative explanation for variable glutamate concentrations between the 3 ApoE transgenic groups is that differences in membrane chemical composition and biophysical properties may be responsible for variable efficiency of glutamate transporters or vesicle

recycling. ApoE is involved in the transport of lipids to regenerating neurons for membrane synthesis (Poirier et al. 1991, Poirier et al. 1993a). Moreover, cholesterol and phospholipid concentrations in the CSF are related to ApoE genotype (Yamauchi et al. 1999) and cholesterol distribution in synaptic membranes is modified in ApoE knock-out mice (Igbavboa et al. 1997). These findings support the view that synaptic membrane composition and biophysical properties may differ between the 3 ApoE transgenic groups, with possible pronounced consequences on synaptic transmission (and presynaptic glutamate concentrations).

Since these relatively aged ApoE transgenic mice have not yet been tested for the detection of cognitive or electrophysiological deficits, it is difficult to draw any conclusions about the functional consequences of differential glutamate immunolabelling. At the presynaptic level, decreased glutamate content intuitively suggests decreased neurotransmission. Electrophysiological experiments should be able to verify this hypothesis. In favour of this argument, presynaptic axons in the spinal cord of the lamprey which are adapted to high level activity, contain higher glutamate concentrations than axons adapted to low-level activity (Shupliakov et al. 1992, Shupliakov et al. 1995, Shupliakov et al. 1997). However, there is an alternative hypothesis that glutamate level in boutons is low as a consequence of intense synaptic activity that depletes the vesicular content in neurotransmitter. Such effects have been previously observed after lesion of the nigrostriatal pathway (Meshul et al. 1999) where a decrease in presynaptic terminal labelling was accompanied by an increase in extracellular glutamate. If this was the case in mice with low glutamate labelling (and low synapse density too), increased synaptic activity could be interpreted as a compensatory mechanism against synaptic loss. Compensatory mechanisms have been described in detail in Barnes' electrophysiological experiments in the hippocampus of aged rats (Barnes 1994). While synaptic numbers decrease with aging in the *MML*, the synaptic strength of the remaining synapses is increased. However, this was shown to be due to greater postsynaptic

responses rather than increase in quantal release of transmitter. These findings therefore argue in favour of poor neurotransmission in mice with poor glutamate immunogold labelling.

The glutamate pool measured in dendritic profiles also varied with ApoE genotype. This may reflect various degrees of cellular activity or cellular 'health'. Low concentrations of glutamate in dendrites may be associated with reduced local dendritic protein synthesis. Incidentally, long term potentiation, which is considered as a model for the cellular changes associated with memory formation (Bliss & Collindridge 1993), is dependent on protein synthesis. Thus it is conceivable that alteration of protein synthesis in dendrites may also impair the mechanisms of memory formation in the hippocampus.

Whether similar ApoE-dependent glutamate concentrations can be observed in other hippocampal regions such as CA1, and whether it is associated with behavioural and cognitive deficits remains to be determined. As yet, no major cognitive differences have been reported between ApoE3 and ApoE4 male and young transgenic mice (Raber et al. 1998, Veinbergs et al. 1999).

Other neurotransmitter disorders have been reported in ApoE deficient mice. The cholinergic system is one of the most affected but there seem to be strain differences (Anderson & Higgins 1997, Chapman & Michaelson 1998, Fagan et al. 1998, Gordon et al. 1995, Gordon et al. 1996b, Krzywkowski et al. 1999). Chapman and Michaelson (Chapman & Michaelson 1998) also described decreased synaptic densities for noradrenergic and serotonergic projections, with longer pathways being more affected. Although they interpreted their results as a loss of presynaptic terminals, they measured only the binding of radioligands specific of noradrenergic and serotonergic transporters. This suggests that again, there might be a relationship between ApoE genotype and number of neurotransmitter transporters.

In conclusion, ApoE polymorphism is associated with variable glutamate immunoreactivity in the neuropil of the MML of aged ApoE transgenic mice. Although the mechanism behind this effect is unknown, ApoE role in lipid transport may be associated with variable chemical composition of cellular membrane and may be responsible for reduced neuronal glutamate concentrations. Neurotransmission may be reduced in hApoE3 and hApoE4 mice compared to hApoE2 mice, with possible cognitive and behavioural deficits.

## **Chapter 4 Discussion**

## 4.1 Summary

The aim of this thesis was to determine the influence of ApoE polymorphism on typical synaptic markers of neuropathology, such as synapse number and size, in the DG of human ApoE transgenic mice. Both a longitudinal and cross-sectional analysis of this aging study demonstrated contrasting effects of mouse ApoE, and human ApoE isoforms on synaptic plasticity.

Both WT and ApoE KO mice displayed no major changes in synaptic size or number in the MML of the DG with aging. While such a finding is in agreement with a previous report for WT animals, the lack of effect in ApoE KO mice demonstrates that KO animals sometimes fail to provide informative data about the missing gene. However, other lines of ApoE KO mice used by other groups exhibit synaptic degeneration, suggesting that compensatory mechanisms most likely account for the lack of effect observed here.

In contrast, clear differences appeared during aging between the human ApoE transgenic mice, with opposite patterns of synaptic plasticity in hApoE2 and hApoE4 mice. Whereas hApoE2 mice maintained a high number of synapses of smaller size even late in life, hApoE4 mice seemed unable to do so and displayed severe synaptic loss associated with an increase in synaptic size. This correlation between size and number resembles that observed in aged human and AD patients. However, only in AD patients is there a massive loss of synapses in the MML of the DG, whereas decrease in synapse numbers is not significant in normal elderly individuals. This suggests that the pattern of synaptic changes observed in hApoE4 mice is not only humanised but also closely matches that of AD patients. The cross-sectional study showed that at old age, hApoE4 mice have the least synapses, and hApoE2 mice the most, compared to WT and ApoE KO mice. Moreover, at 17 months there was a trend towards decreased hippocampal size in hApoE4 mice compared to hApoE3 and hApoE2 mice. Such influences of ApoE genotype on hippocampal volume have also been reported in normal

subjects (Tohgi et al. 1997) and AD patients (Geroldi et al. 1999, Lehtovirta et al. 1995), enhancing the similar dependence of a wide range of morphological parameters upon ApoE genotype, both in human ApoE transgenic mice and human individuals. In addition to maintain high synaptic numbers, aged hApoE2 mice also displayed higher glutamate immunogold labelling in presynaptic boutons and dendrites of the MML, compared to age-matched hApoE3 and hApoE4 mice. Immunogold labelling in presynaptic boutons correlated positively with synapse density, suggesting that decreased synaptic transmission may add to synaptic loss in aged hApoE4 mice.

Finally, there was very little difference in hippocampal volume, synapse number and size, and glutamate immunogold labelling in the MML of the DG between hApoE3 and hApoE4 mice at 17 months old. Because the  $\epsilon$ 4 allele is a risk factor for AD and the  $\epsilon$ 3 allele is the most common of all 3 alleles in the general population, most of the literature regarding ApoE genotype focused on those 2 alleles, with very little attention given to  $\epsilon$ 2. In general, opposite effects on various parameters (such as AD pathology, neurite growth, infarct size after ischemia, cognitive abilities) are described for  $\epsilon$ 4 and  $\epsilon$ 3. Although similar results were expected in this thesis, the parameters investigated here failed to distinguish the effects of the  $\epsilon$ 4 and  $\epsilon$ 3 alleles. Seventeen months was the age at which all genotypes were available for the human transgenic mice with the 3 isoforms. The literature generally describes a gradation of effects, with beneficial influences for the  $\epsilon$ 2 allele, a moderate influence for the  $\epsilon$ 3 allele and a deleterious influence for the  $\epsilon$ 4 allele. While marked differences may appear early between the  $\epsilon$ 4 and  $\epsilon$ 2 alleles, because they are both at the end of the spectrum, this may not be the case for the  $\epsilon$ 4 and  $\epsilon$ 3 alleles. It is conceivable that, although hApoE3 and hApoE4 mice provided similar data at 17 months, the same animals would display marked differences later in life. This hypothesis is supported by the observation that even after 17 months of age, synapse numbers continue to decrease by 29% in hApoE4 mice.

## 4.2 Possible mechanisms involved in ApoE-dependent synaptic plasticity during aging

The mechanisms responsible for the various patterns of synaptic plasticity in the different lines of transgenic mice are unclear. Of all the functions described for ApoE in the general introduction, its interactions with A $\beta$ , modulating its clearance, aggregation and toxicity are unlikely to be relevant to the effects observed here. Indeed, there has been no report of A $\beta$  deposition in these human ApoE transgenic mice (Xu et al. 1995, Xu et al. 1996, Xu et al. 1998b, Xu et al. 1999a), or in another construct (Smith et al. 1998). A $\beta$  toxicity modulated by ApoE isoforms (Ma et al. 1996, Miyata & Smith 1996) or direct ApoE toxicity (DeMattos et al. 1999, Jordan et al. 1998, Moulder et al. 1999, Tolar et al. 1997) are also unlikely factors. There were no obvious signs of neuronal loss in the DG or other hippocampal fields in any the human ApoE transgenic lines used here, even in aged animals. However, since synaptic terminals in the MML arise from EC cells (Amaral 1995), it cannot be ruled out that synaptic loss in hApoE4 mice is the result of EC neuron degeneration, as it may be the case in AD (Hyman et al. 1990, Lippa et al. 1992).

Other properties of ApoE could be involved in the differential synaptic plasticity observed here in aged mice. ApoE is known for its role in lipid transport (Mahley 1988). There are good indications that reactive synaptogenesis after ECL involves ApoE-mediated transport of cholesterol in the MML (Fagan et al. 1998, Poirier et al. 1991, Poirier et al. 1993a, Teter et al. 1999b) and that ApoE-induced neurite outgrowth *in vitro* requires lipids (Bellosta et al. 1995, Nathan et al. 1994 but see DeMattos et al. 1998). The involvement of ApoE in the maintenance of synaptic integrity could therefore occurs at several levels:



### *1) Differential lipid content of ApoE-enriched lipoproteins*

Only recent studies have examined the lipid composition of ApoE-enriched lipoproteins produced by astrocytes and present in CSF (Fagan et al. 1999, LaDu et al. 1998). Nascent astrocyte lipoproteins contain little core lipid and are discoidal in shape, whereas CSF lipoproteins are larger spherical particles with cholesterol ester in their core. It was suggested that nascent astrocyte lipoproteins are converted from discs to spherical particles by acquiring cholesterol ester within the parenchyma of the CNS before reaching the CSF (LaDu et al. 1998). In human transgenic mice, ApoE genotype does not influence the lipid content of astrocytic lipoproteins, but lipoprotein lipid secretion (particularly cholesterol) is proportional to the amount of ApoE secreted by astrocytes (Fagan et al. 1999). In contrast, in humans, the CSF of  $\epsilon 2/\epsilon 3$  carriers contains more cholesterol and phospholipids than  $\epsilon 3/\epsilon 3$  carriers, which in turn contains more of these lipids than  $\epsilon 4/\epsilon 3$  individuals (Yamauchi et al. 1999). hApoE2 mice used in this thesis express more ApoE than ApoE4 mice, and thus may produce lipoproteins enriched with more lipids than those of hApoE4 mice, suggesting that lipid-dependent processes, such as neuronal membrane repair, may be affected by ApoE genotype. In support of this view, membrane lipid composition is altered in ApoE KO mice (Igbavboa et al. 1997, Lomnitski et al. 1999) and synaptogenesis after ECL is impaired in ApoE KO mice (Fagan et al. 1998, Poirier et al. 1991, Poirier et al. 1993a, Teter et al. 1999b). Changes in membrane permeability and stability are likely to have consequences on the activity of pre- and post-synaptic proteins such as receptors and neurotransmitter transporters. Increased glutamate immunolabelling in presynaptic boutons and dendrites in hApoE2 mice could therefore be the result of altered glutamate transport at these sites, as suggested in 3.8.

### *2) Differential binding of ApoE-enriched lipoproteins to ApoE receptors*

Of all the ApoE receptors as yet identified, only 2 have been characterised for their binding with the 3 ApoE isoforms: in non neuronal cells, ApoE2 is highly defective in LDL-receptor

binding activity (less than 2% of normal ApoE3 activity) (Weisgraber et al. 1982). In contrast, ApoE3 and ApoE4 show an equivalent ability to enhance binding and stimulate uptake of  $\beta$ -VLDL by the LRP pathway, whereas ApoE2 is only 40% as active (Kowal et al. 1990). Although differential binding to these receptors does not provide evidence of a more efficient role in synaptic maintenance for the ApoE2 isoform compared to ApoE4, other receptors expressed on neurons such as ApoER2 and VLDLr may be involved (Christie et al. 1996, Kim et al. 1996, Page et al. 1998) and need ApoE binding characterisation.

### 3) *Differential intracellular retention*

The first experiments describing the effects of ApoE isoforms on neurite outgrowth also reported differential accumulation of ApoE *in vitro*, with greater intracellular accumulation of ApoE3 (and to a lesser extent ApoE2), compared to ApoE4 (Ji et al. 1998, Nathan et al. 1995, Pitas 1996). This differential retention requires the presence of lipid or a lipoprotein, and is mediated primarily by cell surface HSPG, but does not involve the LDL or LRP receptors. Preferential release of ApoE4 from the cells results in decreased accumulation of ApoE4 (Ji et al. 1998). In the brain of human ApoE transgenic mice, the intensity of ApoE neuronal immunostaining varies with ApoE genotype and is particularly strong in hApoE3 mice. As with human tissue, granule cells of the DG of transgenic mice are generally non-immunoreactive, in contrast with cortical neurons (Xu et al. 1996, Xu et al. 1998b, Xu et al. 1999b, Xu et al. 1999a). Since synapses in the MML relay afferent inputs from the EC to the spines and dendrites of the granule cells, it is more likely that differences in synaptic plasticity are related to variable ApoE expression in EC neurons rather than in DG cells. In addition, several studies also indicate an involvement of ApoE of glial origin in synaptic remodelling (Poirier et al. 1991, Poirier et al. 1993a, Zarow & Victoroff 1998). It would be useful to characterise the precise cellular expression of ApoE in relation to ApoE genotype and cell types. It is well established that lipoproteins internalised via the LDL and LRP receptors follow an endocytic

pathway that leads to lysosomal degradation (Krieger & Herz 1994). However, it is unclear how a portion of ApoE can escape this degradation (DeMattos et al. 1999). One possibility is that ApoE is trapped by cytoplasmic proteins.

#### *4) Differential interaction with cytoskeleton proteins*

*In vitro* studies have shown that ApoE3 binds to the microtubule binding domains of Tau and MAP2c, whereas ApoE4 does not (Huang et al. 1994, Huang et al. 1995, Strittmatter et al. 1994). Moreover, the inhibitory effect of ApoE4 on neurite outgrowth is associated with a disruption of microtubules, as demonstrated by immunocytochemical localisation, biochemical assay and electron microscopy (Nathan et al. 1995). It remains to determine that ApoE3 facilitates microtubule formation and neurite outgrowth *in vivo*, and elucidate the mechanism behind the inhibitory effect of ApoE4. The aged brain is likely to undergo physical and chemical injuries, and therefore to require structural remodelling in neurons. The inhibitory effect of ApoE 4 on neurite outgrowth could explain why  $\epsilon$ 4 AD patients exhibit significantly less plastic dendritic changes in various brain regions compared to non- $\epsilon$ 4 carriers, and suggests a reduced capacity of dendritic reorganisation to compensate neuronal loss (Arendt et al. 1997). Both in humans and rodents, the DG still undergoes neurogenesis that persists but declines with age (Eriksson et al. 1998, Kempermann et al. 1998, Kuhn et al. 1996). This suggests that the dendritic growth during maturation of these new granule cells may be modulated by ApoE genotype, with consequences on the establishment of synaptic circuitry in the ML. The combination of neurogenesis and ApoE4 influence on dendritic growth in aged hApoE4 mice may result in the loss of synapses observed in the MML.

### **4.3 Functional implications on behaviour and cognitive performance in aged individuals**

The DG represents a crucial gate for cortical inputs which are further processed in the hippocampus, and transferred to the neocortex for long-term storage of memory (Eichenbaum et al. 1996). Therefore, disruption of the DG synaptic transmission associated with possession of the  $\epsilon 4$  allele is likely to have consequence on learning and memory in  $\epsilon 4$  individuals. Even in the case of compensatory synaptic enlargement observed in hApoE4 transgenic mice, the loss of fine tuning of specific sets of synapses could affect the way new information is stored or recalled in the hippocampus. If other cortical regions were to be affected by decreased regenerative capacity, more widespread cognitive deficits could result.

Data from clinical studies in non-demented elderly are consistent with this view. Individuals with the  $\epsilon 4$  allele have poorer cognitive abilities and memory than non  $\epsilon 4$  carriers, whereas those with the  $\epsilon 2$  allele perform better than all the other genotypes. In addition, subjects with the  $\epsilon 2$  allele maintain their cognitive performance at follow-up examination, whereas those with other genotypes deteriorate with age (Berr et al. 1996, Bondi et al. 1995, Feskens et al. 1994, Helkala et al. 1995, Helkala et al. 1996, Henderson et al. 1995, Hyman et al. 1996a, Kuller et al. 1998, Reed et al. 1994, Soininen & Riekkinen 1996, Yaffe et al. 1997). Moreover, animal models of head injury and clinical cases provide further evidence that mortality and neurological deficits in survivors are increased in  $\epsilon 4$  individuals (Alberts et al. 1995, Chen et al. 1997, Horsburgh et al. 1999b, Jordan et al. 1997, Laskowitz et al. 1997, Laskowitz et al. 1998, Nicoll et al. 1995, Sheng et al. 1998, Sheng et al. 1999). These data show that inheritance of the  $\epsilon 4$  allele is associated with an increased chance of developing cognitive impairment and dementia, whereas inheritance of the  $\epsilon 2$  allele is associated with a smaller risk for developing cognitive impairment, independent of the risk to develop AD.

Although transgenic studies failed to detect major cognitive impairment in young hApoE4 mice (Raber et al. 1998, Veinbergs et al. 1999), it is likely that behavioural tasks that are dependent on the integrity of the hippocampus, such as the Morris water maze, would detect memory deficits in aged hApoE4 mice.

## 4.4 Conclusions

This thesis demonstrated that possession of the  $\epsilon 4$  allele in human transgenic mice is associated with a pattern of plasticity that is normally developed by AD patients in the MML of the DG in the early stage of the disease. In contrast, possession of the  $\epsilon 2$  allele prevented synaptic loss and was associated with a moderate decrease in synaptic size. These results are in agreement with the general view that the  $\epsilon 4$  allele is deleterious possibly when neuronal repair is required, for example after head injury, during aging, or in AD to counter neurodegeneration induced by A $\beta$  deposition or NFT. Although neuronal repair is the ApoE function favoured to explain differential synaptic plasticity here, other mechanisms such as protection against oxidative insult or modulation of glial response against inflammation cannot be excluded (reviewed in Laskowitz et al. (1998)).

More work is necessary to characterise this construct of human ApoE transgenic mice. However, the set of data presented here suggests that the hApoE4 transgenic model may reflect generally the synaptic pathology occurring in the DG of AD patients. This is promising for the development of better models of AD. So far, only good models of A $\beta$  deposition have been obtained (Duff 1998). However, none of these models lead to synaptic loss, neurodegeneration (but see Calhoun et al. 1998b), or formation of NFT. Yankner's group recently showed that rodents and primates react differently to injection of fibrillar A $\beta$  (Geula et al. 1998). A $\beta$  toxicity and accumulation of phosphorylated Tau were greater in aged rhesus monkeys and not significant in aged rats. The lack of neurofibrillary degeneration in APP transgenic mice may be due to differences between human and murine Tau proteins. In addition, ApoE is normally expressed only in glial cells in rodents whereas it is expressed both in neurons and glia in humans (Boyles et al. 1985, Han et al. 1994a).

Thus transgenic models for human AD may need to incorporate specific properties and cellular localisation of human gene expression in order to model the disease accurately. Multiple transgenic mice expressing human APP, ApoE and Tau isoforms may provide a better resemblance with AD pathology.

## **4.5 Future directions**

Several experiments can be suggested to validate and expand on the results presented here.

### **4.5.1 Quantification of ApoE expression in human ApoE transgenic mice**

One source of concern for the interpretation of these results was the variable level of ApoE expression in the different lines of ApoE transgenic mice. The literature strongly indicates that it is the identity of each ApoE isoform that is relevant to the specific and differential effects observed in experimental situations. However, it cannot be ruled out that variable expression of ApoE protein is partly responsible for the differences in synaptic plasticity during aging observed between hApoE2 and hApoE4 mice. In addition, it is not known if ApoE expression varies with increasing age, and if this variation is affected by ApoE genotype. Therefore, the quantitation of ApoE expression in the brain of these human ApoE transgenic mice is essential to permit detailed interpretation of the results presented here. Western blots could be used to examine global expression in the brain and in the hippocampus. In addition, precise ApoE expression and cellular distribution could be quantified by immunogold labelling, in the same way that relative glutamate concentrations were estimated in section 3.8.

### **4.5.2 Quantification of EC neurons number in human ApoE transgenic mice**

One hypothesis put forward in section 4.2 was that synaptic loss occurring in the MML of aged hApoE4 mice is the result of EC neuron degeneration. This could be tested by counting EC neurons for each human ApoE transgenic line. The synapse per EC neuron ratio could then be calculated and compared between lines. The relationship between EC neuron numbers and synapse density and their variation with age would provide some information about which of these 2 parameters is affected first and if they vary to the same extent.



#### **4.5.3 Assessment of dendritic integrity in DG granule cells of human ApoE transgenic mice**

As an alternative, or in addition to EC cell loss in hApoE4 mice, altered dendritic branching of DG granule cells may be responsible for the decrease in synapse number. Arendt (Arendt et al. 1997) demonstrated that dendritic growth is severely deficient in other neuronal populations than the DG in  $\epsilon$ 4 AD patients compared to non- $\epsilon$ 4 carriers. This suggests that DG granule cells in hApoE4 mice may similarly be affected. The pattern of dendritic branching could be assessed by stereological methods applied to Golgi-stained preparations.

#### **4.5.4 Assessment of cholinergic integrity in the hippocampus of human ApoE transgenic mice**

The cholinergic system is dependent on lipid delivery for synthesis of ACh (Nitsch et al. 1992).  $\epsilon$ 4 AD patients show a reduced activity of the enzyme responsible for the synthesis of ACh, ChAT, reduced nicotinic receptor binding sites (Arendt et al. 1997, Poirier et al. 1995 but see Svensson et al. 1997), and reduced number of cholinergic neurons in the nucleus basalis of Meynert compared to non- $\epsilon$ 4 carriers (Arendt et al. 1997, Poirier et al. 1995 but see Salehi et al. 1998). To test whether these human ApoE transgenic mice provide a good model of cholinergic status in humans, some of the parameters mentioned above could be assessed. The DG and particularly its IML receive abundant cholinergic projections from the septum and the diagonal band of Broca (Amaral 1995). In normal aged individuals and some AD patients, this region is very reactive to EC deafferentation and responds with compensatory synaptogenesis extending across the ML (Deller & Frotscher 1997, Geddes et al. 1985, Hyman et al. 1987a). It was suggested that  $\epsilon$ 4 carriers are impaired for such reactive plasticity. Estimation of synapse density in the IML and ChAT/AChE (two enzymes involved in ACh metabolism) immunogold labelling quantitation could test this hypothesis.

#### **4.5.5 Study of the influence of ApoE polymorphism on synaptic transmission and cognitive performance in hApoE mice**

The main result of this thesis is the inability of aged hApoE4 transgenic mice to maintain a constant synapse per neuron ratio in the MML, in contrast to WT, ApoE KO and hApoE2 mice. The consequences of such effects are still unknown and could be examined with electrophysiological and behavioural experiments. Several parameters of synaptic transmission could be determined in the MML by stimulating the PP. LTP of the PP is another model commonly used to assess the potential for synaptic plasticity at the synaptic level. From a behavioural point of view, several hippocampal-dependent learning tasks such as the Morris water maze provide information about cognitive status. Such experiments have already been carried out in the same mice as those used in this thesis (Veinbergs et al. 1999) and in another construct (Raber et al. 1998), but at an early age point (3-12 months) when synapse numbers have not yet declined in hApoE4 mice. Ideally, experiments should be carried out at a young and aged time-points to establish potential cognitive impairment, and examine their correlation with synapse numbers in the DG or other regions. It would be interesting to determine if there is great inter-individual variability of cognitive decline in aged mice, as there is in rats, monkeys and humans (Rapp & Amaral 1992, Rapp & Gallagher 1996, Rasmussen et al. 1996).

## **Chapter 5   References**

Adams, I. 1987: Plasticity of the Synaptic Contact Zone Following Loss of Synapses in the Cerebral-Cortex of Aging Humans. *Brain Research* **424**, 343-351.

Adams, J. H. & Duchen, L. W. 1992: Greenfield's neuropathology.

Akaaboune, M., Villanova, M., Festoff, B. W., Verdieresahuque, M. & Hantai, D. 1994: Apolipoprotein-E Expression At Neuromuscular-Junctions in Mouse, Rat and Human Skeletal-Muscle. *Febs Letters* **351**, 246-248.

Alberts, M. J., Graffagnino, C., McClenny, C., Delong, D., Strittmatter, W., Saunders, A. M. & Roses, A. D. 1995: ApoE Genotype and Survival From Intracerebral Hemorrhage. *Lancet* **346**, 575.

Amaral, D. G. & Witter, M. P. 1989: The 3-Dimensional Organization of the Hippocampal-Formation - a Review of Anatomical Data. *Neuroscience* **31**, 571-591.

Amaral, D. G. & Witter, M. P. 1995: Hippocampal formation. In: The Rat Nervous System. Academic Press. pp. 443-493.

Anderson, R., Barnes, J. C., Bliss, T., Cain, D. P., Cambon, K., Davies, H. A., Errington, M. L., Fellows, L. A., Gray, R. A., Hoh, T., Stewart, M., Large, C. H. & Higgins, G. A. 1998: Behavioural, physiological and morphological analysis of a line of apolipoprotein E knockout mouse. *Neuroscience* **85**, 93-110.

Anderson, R. & Higgins, G. A. 1997: Absence of central cholinergic deficits in ApoE knockout mice. *Psychopharmacology* **132**, 135-144.

Anderton, B. H. 1997: Changes in the ageing brain in health and disease. *Philosophical transactions of the Royal Society London B* **352**, 1781-1792.

Arendt, T., Schindler, C., Bruckner, M. K., Eschrich, K., Bigl, V., Zedlick, D. & Marcova, L. 1997: Plastic neuronal remodeling is impaired in patients with Alzheimer's disease carrying apolipoprotein epsilon 4 allele. *Journal Of Neuroscience* **17**, 516-529.

Aronica, E., Dickson, D. W., Kress, Y., Morrison, J. H. & Zukin, R. S. 1998: Non-plaque dystrophic dendrites in Alzheimer hippocampus: a new pathological structure revealed by glutamate receptor immunocytochemistry. *Neuroscience* **82**, 979-991.

Arriagada, P. V., Growdon, J. H., Hedleywhyte, E. T. & Hyman, B. T. 1992: Neurofibrillary Tangles But Not Senile Plaques Parallel Duration and Severity of Alzheimers-Disease. *Neurology* **42**, 631-639.

Azher, S. N., Doody, R. S., Haykal, H. A., Dunn, J. K., Liao, T. Y. & Schneider, L. 1999: Does Apo epsilon 4 correlate with MRI changes in Alzheimer's disease? *Neurology* **52**, A366.

Bailey, C. H. & Kandel, E. R. 1993: Structural changes accompanying memory storage. *Annual review of physiology* **55**, 397-426.

Bales, K. R., Verina, T., Dodel, R. C., Du, Y. S., Altstiel, L., Bender, M., Hyslop, P., Johnstone, E. M., Little, S. P., Cummins, D. J., Piccardo, P., Ghetti, B. & Paul, S. M. 1997: Lack of apolipoprotein E dramatically reduces amyloid beta-peptide deposition. *Nature Genetics* **17**, 263-264.

Barnes, C. A. 1994: Normal Aging - Regionally Specific Changes in Hippocampal Synaptic Transmission. *Trends in Neurosciences* **17**, 13-18.

Barnes, C. A. 1999: Do synaptic markers provide a window on synaptic effectiveness in the aged hippocampus? *Neurobiology of Aging* **20**, 349-351.

- Beffert, U., Aumont, N., Dea, D., LussierCacan, S., Davignon, J. & Poirier, J. 1998a: beta-amyloid peptides increase the binding and internalization of apolipoprotein E to hippocampal neurons. *Journal Of Neurochemistry* **70**, 1458-1466.
- Beffert, U., Cohn, J. S., PetitTurcotte, C., Tremblay, M., Aumont, N., Ramassamy, C., Davignon, J. & Poirier, J. 1999: Apolipoprotein E and beta-amyloid levels in the hippocampus and frontal cortex of Alzheimer's disease subjects are disease-related and apolipoprotein E genotype dependent. *Brain Research* **843**, 87-94.
- Beffert, U., Danik, M., Krzywkowski, P., Ramassamy, C., Berrada, F. & Poirier, J. 1998b: The neurobiology of apolipoproteins and their receptors in the CNS and Alzheimer's disease. *Brain Research Reviews* **27**, 119-142.
- Beffert, U. & Poirier, J. 1996: Apolipoprotein E, plaques, tangles and cholinergic dysfunction in Alzheimer's disease. *Annals Of The New York Academy Of Sciences* **777**, 166-174.
- Bellosta, S., Nathan, B. P., Orth, M., Dong, L. M., Mahley, R. W. & Pitas, R. E. 1995: Stable expression and secretion of apolipoproteins e3 and e4 in mouse neuroblastoma-cells produces differential-effects on neurite outgrowth. *Journal Of Biological Chemistry* **270**, 27063-27071.
- Berg, L., McKeel, D. W., Miller, J. P., Storandt, M., Rubin, E. H., Morris, J. C., Baty, J., Coats, M., Norton, J., Goate, A. M., Price, J. L., Gearing, M., Mirra, S. S. & Saunders, A. M. 1998: Clinicopathologic studies in cognitively healthy aging and Alzheimer disease - Relation of histologic markers to dementia severity, age, sex, and apolipoprotein E genotype. *Archives of Neurology* **55**, 326-335.
- Berr, C., Dufouil, C., Brousseau, T., Richard, F., Amouyel, P., Marceteau, E. & Alperovitch, A. 1996: Early effect of ApoE-epsilon 4 allele on cognitive results in a group of highly performing subjects: The EVA study. *Neuroscience Letters* **218**, 9-12.
- Berr, C., Hauw, J. J., Delacre, P., Duyckaerts, C. & Amouyel, P. 1994: Apolipoprotein-E Allele Epsilon-4 Is Linked to Increased Deposition of the Amyloid Beta-Peptide (a-Beta) in Cases With or Without Alzheimers-Disease. *Neuroscience Letters* **178**, 221-224.
- Bertoni Freddari, C., Fattoretti, P., Casoli, T., Caselli, U. & MeierRuge, W. 1996a: Deterioration threshold of synaptic morphology in aging and senile dementia of Alzheimer's type. *Analytical and Quantitative Cytology and Histology* **18**, 209-213.
- Bertoni Freddari, C., Fattoretti, P., Casoli, T., MeierRuge, W. & Ulrich, J. 1990: Morphological Adaptive Response of the Synaptic Junctional Zones in the Human Dentate Gyrus During Aging and Alzheimers-Disease. *Brain Research* **517**, 69-75.
- Bertoni Freddari, C., Fattoretti, P., Paoloni, R., Caselli, U., Galeazzi, L. & MeierRuge, W. 1996b: Synaptic structural dynamics and aging. *Gerontology* **42**, 170-180.
- Bertoni Freddari, C., Giuli, C., Pieri, C. & Paci, D. p. 1986: Quantitative investigation of the morphological plasticity of synaptic junctions in rat dentate gyrus during aging. *Brain research* **366**, 187-192.
- Bertrand, P., Poirier, J., Oda, T., Finch, C. E. & Pasinetti, G. M. 1995: Association of apolipoprotein-e genotype with brain levels of apolipoprotein-e and apolipoprotein j(clusterin) in alzheimer-disease. *Molecular Brain Research* **33**, 174-178.
- Bickeboller, H., Campion, D., Brice, A., Amouyel, P., Hannequin, D., Didierjean, O., Penet, C., Martin, C., PerezTur, J., Michon, A., Dubois, B., Ledoze, F., ThomasAnterion, C., Pasquier, F., Puel, M., Demonet, J. F., Moreaud, O., Babron, M. C., Meulien, D., Guez, D., ChartierHarlin, M. C., Frebourg, T., Agid, Y., Martinez, M. & ClergetDarpoux, F. 1997: Apolipoprotein E and Alzheimer disease: Genotype-specific risks by age and sex. *American Journal of Human Genetics* **60**, 439-446.
- Blennow, K., Bogdanovic, N., Alafuzoff, I., Ekman, R. & Davidsson, P. 1996: Synaptic pathology in alzheimers-disease - relation to severity of dementia, but not to senile plaques, neurofibrillary tangles, or the apoe4 allele. *Journal Of Neural Transmission* **103**, 603-618.

- Blennow, K., Hesse, C. & Fredman, P. 1994: Cerebrospinal-Fluid Apolipoprotein-E Is Reduced in Alzheimers-Disease. *Neuroreport* **5**, 2534-2536.
- Bliss, T. V. P. & Collindridge, G. L. 1993: A synaptic model of memory: long term potentiation in the hippocampus. *Nature* **361**, 31-39.
- Bobinski, M., DeLeon, M. J., Wegiel, J., Desanti, S., Convit, A., SaintLouis, L. A., Rusinek, H. & Wisniewski, H. M. 2000: The histological validation of post mortem magnetic resonance imaging-determined hippocampal volume in Alzheimer's disease. *Neuroscience* **95**, 721-725.
- Bobinski, M., Wegiel, J., Wisniewski, H. M., Tamawski, M., Bobinska, M., Reisberg, B., Mlodzik, B., Deleon, M. & Miller, D. C. 1995a: Neuronal Loss in Hippocampal-Formation Subdivisions Correlates With Duration and Stage of Alzheimer-Disease. *Journal of Neuropathology and Experimental Neurology* **54**, 428.
- Bobinski, M., Wegiel, J., Wisniewski, H. M., Tarnawski, M., Bobinski, M., Reisberg, B., DeLeon, M. J. & Miller, D. C. 1996: Neurofibrillary pathology - Correlation with hippocampal formation atrophy in Alzheimer disease. *Neurobiology Of Aging* **17**, 909-919.
- Bobinski, M., Wegiel, J., Wisniewski, H. M., Tarnawski, M., Reisberg, B., Mlodzik, B., Deleon, M. J. & Miller, D. C. 1995b: Atrophy of hippocampal-formation subdivisions correlates with stage and duration of alzheimer-disease. *Dementia* **6**, 205-210.
- Bondi, M. W., Salmon, D. P., Monsch, A. U., Galasko, D., Butters, N., Katzman, R. & Thal, L. J. 1995: Episodic Memory Changes Are Associated With the Apoe-Epsilon-4 Allele in Nondemented Older Adults. *Neurology* **45**, A373.
- Bontempi, B., LaurentDemir, C., Destrade, C. & Jaffard, R. 1999: Time-dependent reorganization of brain circuitry underlying long-term memory storage. *Nature* **400**, 671-675.
- Bowman, B. H., Jansen, L., Yang, F. M., Adrian, G. S., Zhao, M., Atherton, S. S., Buchanan, J. M., Greene, R., Walter, C., Herbert, D. C., Weaker, F. J., Chiodo, L. K., KaganHallet, K. & Hixson, J. E. 1995: Discovery of a brain promoter from the human transferrin gene and its utilization for development of transgenic mice that express human apolipoprotein E alleles. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 12115-12119.
- Bowman, B. H., Yang, F. M., Buchanan, J. M., Adrian, G. S., Martinez, A. O., Jansen, L., Zhao, M., Atherton, S. L. & Hixson, J. E. 1996: Human APOE protein localized in brains of transgenic mice. *Neuroscience Letters* **219**, 57-59.
- Boyles, J. K., Pitas, R. E., Wilson, E., Mahley, R. W. & Taylor, J. M. 1985: Apolipoprotein-E Associated With Astrocytic Glia of the Central Nervous-System and With Nonmyelinating Glia of the Peripheral Nervous-System. *Journal of Clinical Investigation* **76**, 1501-1513.
- Boyles, J. K., Zoellner, C. D., Anderson, L. J., Kosik, L. M., Pitas, R. E., Weisgraber, K. H., Hui, D. Y., Mahley, R. W., Gebickehaerter, P. J., Ignatius, M. J. & Shooter, E. M. 1989: A Role For Apolipoprotein-E, Apolipoprotein-a-I, and Low-Density Lipoprotein Receptors in Cholesterol Transport During Regeneration and Remyelination of the Rat Sciatic-Nerve. *Journal of Clinical Investigation* **83**, 1015-1031.
- Braak, H. & Braak, E. 1991: Neuropathological Staging of Alzheimer-Related Changes. *Acta Neuropathologica* **82**, 239-259.
- Braak, H. & Braak, E. 1998: Evolution of neuronal changes in the course of Alzheimer's disease. *Journal of Neural Transmission-Supplement*, 127-140.
- Bramham, C. R., Torp, R., Zhang, N., Storm Mathisen, J. & Ottersen, O. P. 1990: Distribution of Glutamate-Like Immunoreactivity in Excitatory Hippocampal Pathways - a Semiquantitative Electron-Microscopic Study in Rats. *Neuroscience* **39**, 405-417.

- Brody, H. 1955: Organization of the cerebral cortex. III. A study of aging in the human cerebral cortex. *Journal of comparative neurology* **102**, 511-556.
- Buttini, M., Orth, M., Bellosta, S., Akcefe, H., Pitas, R. E., WyssCoray, T., Mucke, L. & Mahley, R. W. 1999: Expression of human apolipoprotein E3 or E4 in the brains of Apoe(-/-) mice: Isoform-specific effects on neurodegeneration. *Journal of Neuroscience* **19**, 4867-4880.
- Cabalka, L. M., Hyman, B. T., Goodlett, C. R., Ritchie, T. C. & Vanhoesen, G. W. 1992: Alteration in the Pattern of Nerve-Terminal Protein Immunoreactivity in the Perforant Pathway in Alzheimers-Disease and in Rats After Entorhinal Lesions. *Neurobiology of Aging* **13**, 283-291.
- Calhoun, M. E., Kurth, D., Phinney, A. L., Long, J. M., Hengemihle, J., Mouton, P. R., Ingram, D. K. & Jucker, M. 1998a: hippocampal neuron and synaptophysin-positive bouton number in aging C57BL/6 mice. *Neurobiol. Aging* **19**, 599-606.
- Calhoun, M. E., Wiederhold, K. H., Abramowski, D., Phinney, A. L., Probst, A., SturchlerPierrat, C., Staufenbiel, M., Sommer, B. & Jucker, M. 1998b: Neuron loss in APP transgenic mice. *Nature* **395**, 755-756.
- Callahan, L. M. & Coleman, P. D. 1995: Neurons bearing neurofibrillary tangles are responsible for selected synaptic deficits in alzheimers-disease. *Neurobiology Of Aging* **16**, 311-314.
- Castano, E. M., Prelli, F., Wisniewski, T., Golabek, A., Kumar, R. A., Soto, C. & Frangione, B. 1995: Fibrillogenesis in Alzheimers-Disease of Amyloid-Beta Peptides and Apolipoprotein-E. *Biochemical Journal* **306**, 599-604.
- Cavalieri, B. (1635): *Geometria Indivisibilibus Continuatorum. Typis Clemetis Feronij. Bononi.: Reprinted (1966) as Geometria degli Indivisibili.*, Unione Tipographico-Editrice Torinese, Torino.
- Chapman, S. & Michaelson, D. M. 1998: Specific neurochemical derangements of brain projecting neurons in apolipoprotein E-deficient mice. *Journal of Neurochemistry* **70**, 708-714.
- Chartierharlin, M. C., Crawford, F., Houlden, H., Warren, A., Hughes, D., Fidani, L., Goate, A., Rossor, M., Roques, P., Hardy, J. & Mullan, M. 1991: Early-Onset Alzheimers-Disease Caused By Mutations At Codon-717 of the Beta-Amyloid Precursor Protein Gene. *Nature* **353**, 844-846.
- Chen, Y., Lonnitski, L., Michaelson, D. M. & Shohami, E. 1997: Motor and cognitive deficits in apolipoprotein E-deficient mice after closed head injury. *Neuroscience* **80**, 1255-1262.
- Christie, R. H., Chung, H., Rebeck, G. W., Strickland, D. & Hyman, B. T. 1996: Expression of the very low-density lipoprotein receptor (VLDL-r), an apolipoprotein-E receptor, in the central nervous system and in Alzheimer's disease. *Journal of Neuropathology and Experimental Neurology* **55**, 491-498.
- Coleman, P. D., Flood, D. G. & West, M. J. 1987: Volumes of the Components of the Hippocampus in the Aging F344 Rat. *Journal of Comparative Neurology* **266**, 300-306.
- Corder, E. H., Saunders, A. M., Risch, N. J., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Rimmler, J. B., Locke, P. A., Conneally, P. M., Schmechel, K. E., Small, G. W., Roses, A. D., Haines, J. L. & Pericakvance, M. A. 1994: Protective effect of apolipoprotein-e type-2 allele for late-onset alzheimer-disease. *Nature Genetics* **7**, 180-184.
- Corder, E. H., Saunders, A. M., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Rimmler, J. B., Locke, P. A., Conneally, P. M., Schmechel, K. E., Tanzi, R. E., Gusella, J. F., Small, G. W., Roses, A. D., Pericakvance, M. A. & Haines, J. L. 1995a: Apolipoprotein-e, survival in alzheimers-disease patients, and the competing risks of death and alzheimers-disease. *Neurology* **45**, 1323-1328.
- Corder, E. H., Saunders, A. M., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Roses, A. D., Pericakvance, M. A., Small, G. W. & Haines, J. L. 1995b: The apolipoprotein-e e4 allele and sex-specific risk of Alzheimer's-disease. *Journal Of The American Medical Association* **273**, 373-374.

- Corder, E. H., Saunders, A. M., Strittmatter, W. J., Schmechel, D. E., Gaskell, P. C., Small, G. W., Roses, A. D., Haines, J. L. & Pericakvance, M. A. 1993: Gene dose of apolipoprotein-e type-4 allele and the risk of Alzheimer's-disease in late-onset families. *Science* **261**, 921-923.
- Cowburn, R., Hardy, J., Roberts, P. & Briggs, R. 1988: Presynaptic and Postsynaptic Glutamatergic Function in Alzheimers- Disease. *Neuroscience Letters* **86**, 109-113.
- Crawley, J. N. 1996: Unusual behavioral phenotypes of inbred mouse strains - Commentary. *Trends in Neurosciences* **19**, 181-182.
- Cummings, B. J., Head, E., Afagh, A. J., Milgram, N. W. & Cotman, C. W. 1996: beta-amyloid accumulation correlates with cognitive dysfunction in the aged canine. *Neurobiology of Learning and Memory* **66**, 11-23.
- Davies, D. C., Horwood, N., Isaacs, S. L. & Mann, D. M. A. 1992: The Effect of Age and Alzheimers-Disease On Pyramidal Neuron Density in the Individual Fields of the Hippocampal-Formation. *Acta Neuropathologica* **83**, 510-517.
- Davies, P. & Maloney, A. J. R. 1976: Selective loss of central cholinergic neurons in Alzheimer's disease. *Lancet* **ii**, 1403.
- De Groot, D. M. G. 1988: Comparison of methods for the estimation of the thickness of ultrathin tissue sections. *Journal of Microscopy* **151**, 23-42.
- DeGroot, D. M. G., Bierman, E. P. B., Bruijnzeel, P. L. B. & Woutersen, R. A. 1995: The 'disector' - A tool for quantitative assessment of synaptic plasticity an example on hippocampal synapses and synapse- perforations in ageing rats13. *European Journal of Morphology* **33**, 305-309.
- DeKosky, S. T. & Scheff, S. W. 1990: Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. *Annals of Neurology* **27**, 457-464.
- Deller, T. & Frotscher, M. 1997: Lesion-induced plasticity of central neurons: Sprouting of single fibres in the rat hippocampus after unilateral entorhinal cortex lesion. *Progress in Neurobiology* **53**, 687-727.
- DeMattos, R. B., Curtiss, L. K. & Williams, D. L. 1998: A minimally lipidated form of cell-derived apolipoprotein E exhibits isoform-specific stimulation of neurite outgrowth in the absence of exogenous lipids or lipoproteins. *Journal of Biological Chemistry* **273**, 4206-4212.
- DeMattos, R. B., Thorngate, F. E. & Williams, D. L. 1999: A test of the cytosolic apolipoprotein E hypothesis fails to detect the escape of apolipoprotein E from the endocytic pathway into the cytosol and shows that direct expression of apolipoprotein E in the cytosol is cytotoxic. *Journal Of Neuroscience* **19**, 2464-2473.
- Desmond, N. L. & Levy, W. B. 1997: Ovarian steroidal control of connectivity in the female hippocampus: An overview of recent experimental findings and speculations on its functional consequences. *Hippocampus* **7**, 239-245.
- De Toledo Morrell, L., Sullivan, M. P., Morrell, F., Wilson, R. S., Bennett, D. A. & Spencer, S. 1997: Alzheimer's disease: In vivo detection of differential vulnerability of brain regions. *Neurobiology Of Aging* **18**, 463-468.
- Dickson, D. W., Crystal, H. A., Bevona, C., Honer, W., Vincent, I. & Davies, P. 1995: Correlations of Synaptic and Pathological Markers With Cognition of the Elderly. *Neurobiology of Aging* **16**, 285-298.
- Diedrich, J. F., Minnigan, H., Carp, R. I., Whitaker, J. N., Race, R., Frey, W. & Haase, A. T. 1991: Neuropathological Changes in Scrapie and Alzheimer's-Disease Are Associated With Increased Expression of Apolipoprotein-E and Cathepsin-D in Astrocytes. *Journal of Virology* **65**, 4759-4768.
- Doubell, T. P. & Stewart, M. G. 1993: Short-term changes in the numerical density of synapses in the intermediate and medial hyperstriatum ventrale following one-trial passive avoidance training in the chick. *Journal of neuroscience* **13**, 2230-2236.



- Dowson, J. H. 1982: Neuronal Lipofuscin Accumulation in Aging and Alzheimer Dementia - a Pathogenic Mechanism. *British Journal of Psychiatry* **140**, 142-148.
- Duff, K. 1998: Recent work on Alzheimer's disease transgenics. *Current Opinion in Biotechnology* **9**, 561-564.
- Eichenbaum, H., Schoenbaum, G., Young, B. & Bunsey, M. 1996: Functional organisation of the hippocampal memory system. *Proceedings of the National Academy of Sciences USA* **93**, 13500-13507.
- Einstein, G., Patel, V., Bautista, P., Kenna, M., McLone, L., Fader, R., Karson, K., Mann, S., Saunders, A. M., Hulette, C., Mash, D., Roses, A. D. & Schmechel, D. E. 1998: Intraneuronal ApoE in human visual cortical areas reflects the staging of Alzheimer disease pathology. *Journal Of Neuropathology And Experimental Neurology* **57**, 1190-1201.
- Elshourbagy, N. A., Liao, W. S., Mahley, R. W. & Taylor, J. M. 1985: Apolipoprotein-E Messenger-Rna Is Abundant in the Brain and Adrenals, As Well As in the Liver, and Is Present in Other Peripheral-Tissues of Rats and Marmosets. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 203-207.
- Erecinska, M. & Silver, I. A. 1990: Metabolism and Role of Glutamate in Mammalian Brain. *Progress in Neurobiology* **35**, 245-296.
- Eriksson, S. E., Perfilieva, E., Bjork-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A. & Gage, F. H. 1998: Neurogenesis in the adult human hippocampus. *Nature Medicine* **4**, 1313-1317.
- Esch, F. S., Keim, P. S., Beattie, E. C., Blacher, R. W., Culwell, A. R., Oltersdorf, T., McClure, D. & Ward, P. J. 1990: Cleavage of Amyloid-Beta Peptide During Constitutive Processing of Its Precursor. *Science* **248**, 1122-1124.
- Esiri, M. M. & Morris, J. H. 1997: The neuropathology of dementia. Cambridge University Press, Cambridge.
- Evans, D. A., Funkenstein, H. & Albert, M. S. 1989: Prevalence of Alzheimer's disease in a community population of older persons; higher than previously reported. *JAMA* **262**, 2551-2556.
- Fagan, A. M., Bu, G. J., Sun, Y. L., Daugherty, A. & Holtzman, D. M. 1996: Apolipoprotein E-containing high density lipoprotein promotes neurite outgrowth and is a ligand for the low density lipoprotein receptor- related protein. *Journal of Biological Chemistry* **271**, 30121-30125.
- Fagan, A. M., Holtzman, D. M., Munson, G., Mathur, T., Schneider, D., Chang, L. K., Getz, G. S., Reardon, C. A., Lukens, J., Shah, J. A. & LaDu, M. J. 1999: Unique lipoproteins secreted by primary astrocytes from wild type, apoE (-/-), and human apoE transgenic mice. *Journal of Biological Chemistry* **274**, 30001-30007.
- Fagan, A. M., Murphy, B. A., Patel, S. N., Kilbridge, J. F., Mobley, W. C., Bu, G. J. & Holtzman, D. M. 1998: Evidence for normal aging of the septo-hippocampal cholinergic system in apoE (-/-) mice but impaired clearance of axonal degeneration products following injury. *Experimental Neurology* **151**, 314-325.
- Farrer, L. A., Cupples, L. A., Haines, J. L., Hyman, B., Kukull, W. A., Mayeux, R., Myers, R. H., PericakVance, M. A., Risch, N. & vanDuijn, C. M. 1997: Effects of age, sex, and ethnicity on the association between apolipoprotein E genotype and Alzheimer disease - A meta-analysis. *Journal Of The American Medical Association* **278**, 1349-1356.
- Fernandez, G., Effern, A., Grunwald, T., Pezer, N., Lehnertz, K., Dumpelmann, M., VanRoost, D. & Elger, C. E. 1999: Real-time tracking of memory formation in the human rhinal cortex and hippocampus. *Science* **285**, 1582-1585.
- Feskens, E. J. M., Havekes, L. M., Kalmijn, S., Deknijff, P., Launer, L. J. & Kromhout, D. 1994: Apolipoprotein E4 Allele and Cognitive Decline in Elderly Men. *British Medical Journal* **309**, 1202-1206.
- Fewster, P. H., Griffin-Brooks, S., Mac Gregor, J. A., Ojalvo-Rose, E. & Ball, M. J. 1991: A topographical pathway by which histopathological lesions disseminate through the brain of patients with Alzheimer's disease. *Dementia* **2**, 121-132.

- Finch, C. E. & Sapolsky, R. M. 1999: The evolution of Alzheimer disease, the reproductive schedule, and apoE isoforms. *Neurobiology of Aging* **20**, 407-428.
- Frisoni, G. B., Govoni, S., Geroldi, C., Bianchetti, A., Calabresi, L., Franceschini, G. & Trabucchi, M. 1995: Gene Dose of the Epsilon-4 Allele of Apolipoprotein-E and Disease Progression in Sporadic Late-Onset Alzheimer's-Disease. *Annals of Neurology* **37**, 596-604.
- Gandy, S., Stafstrom, C., Turner, B., Sweeney, D., Breslow, J., Greengard, P., Smith, J. & Ouimet, C. 1995: Aged apoE deficient mice: abnormal learning and glial markers but no obvious neuropathology. *Soc. for Neurosc. Abstr.* **21**, 5.
- Geddes, J. W., Monaghan, D. T., Cotman, C. W., Lott, I. T., Kim, R. C. & Chui, H. C. 1985: Plasticity of Hippocampal Circuitry in Alzheimers-Disease. *Science* **230**, 1179-1181.
- Geinisman, Y. & Bondareff, W. 1976: Decrease in the number of synapses in the senescent brain: a quantitative electron microscopic analysis of the dentate gyrus molecular layer in the rat. *Mechanisms of ageing and development* **5**, 11-23.
- Geinisman, Y., Bondareff, W. & Dodge, J. T. 1977: Partial deafferentation of neurons in the dentate gyrus of the senescent rat. *Brain research* **134**, 541-545.
- Geinisman, Y., De Toledo Morrell, L. & Morrell, F. 1991: Induction of Long-Term Potentiation Is Associated With an Increase in the Number of Axospinous Synapses With Segmented Postsynaptic Densities. *Brain Research* **566**, 77-88.
- Geinisman, Y., De Toledo Morrell, L., Morrell, F., Persina, I. S. & Rossi, M. 1992: Age-Related Loss of Axospinous Synapses Formed By 2 Afferent Systems in the Rat Dentate Gyrus As Revealed By the Unbiased Stereological Dissector Technique. *Hippocampus* **2**, 437-444.
- Gerlai, R. 1996a: Gene targeting in neuroscience: The systemic approach - Reply. *Trends in Neurosciences* **19**, 188-189.
- Gerlai, R. 1996b: Gene-targeting studies of mammalian behavior: Is it the mutation or the background genotype? *Trends in Neurosciences* **19**, 177-181.
- Geroldi, C., Pihlajamaki, M., Laakso, M. P., DeCarli, C., Beltramello, A., Bianchetti, A., Soininen, H., Trabucchi, M. & Frisoni, G. B. 1999: APOE-epsilon 4 is associated with less frontal and more medial temporal lobe atrophy in AD. *Neurology* **53**, 1825-1832.
- Geula, C., Wu, C., Saroff, D., Lorenzo, A., Yuan, M. & Yankner, B. 1998: Aging renders the brain vulnerable to amyloid beta-protein neurotoxicity. *Nature Medicine* **4**, 827-831.
- Ghebremedhin, E., Schultz, C., Braak, E. & Braak, H. 1998: High frequency of apolipoprotein E epsilon 4 allele in young individuals with very mild Alzheimer's disease-related neurofibrillary changes. *Experimental Neurology* **153**, 152-155.
- Gibson, P. H. 1983: Em Study of the Numbers of Cortical Synapses in the Brains of Aging People and People With Alzheimer-Type Dementia. *Acta Neuropathologica* **62**, 127-133.
- Glenner, G. G. & Wong, C. W. 1984: Alzheimers-Disease and Downs-Syndrome - Sharing of a Unique Cerebrovascular Amyloid Fibril Protein. *Biochemical and Biophysical Research Communications* **122**, 1131-1135.
- Goate, A., Chartierharlin, M. C., Mullan, M., Brown, J., Crawford, F., Fidani, L., Giuffra, L., Haynes, A., Irving, N., James, L., Mant, R., Newton, P., Rooke, K., Roques, P., Talbot, C., Pericakvance, M., Roses, A., Williamson, R., Rossor, M., Owen, M. & Hardy, J. 1991: Segregation of a Missense Mutation in the Amyloid Precursor Protein Gene With Familial Alzheimers-Disease. *Nature* **349**, 704-706.
- Goedert, M. 1993: Tau protein and the neurofibrillary pathology of Alzheimer's disease. *Trends in neuroscience* **16**, 460-465.

- Golomb, J., DeLeon, M. J., Kluger, A., George, A. E., Tarshish, C. & Ferris, S. H. 1993: Hippocampal Atrophy in Normal Aging - an Association With Recent Memory Impairment. *Archives of Neurology* **50**, 967-973.
- Gomez Isla, T., Hollister, R., West, H., Mui, S., Growdon, J. H., Petersen, R. C., Parisi, J. E. & Hyman, B. T. 1997: Neuronal loss correlates with but exceeds neurofibrillary tangles in Alzheimer's disease. *Annals of Neurology* **41**, 17-24.
- Gomez Isla, T., Price, J. L., McKeel, D. W., Morris, J. C., Growdon, J. H. & Hyman, B. T. 1996: Profound loss of layer II entorhinal cortex neurons occurs in very mild Alzheimer's disease. *Journal of Neuroscience* **16**, 4491-4500.
- Gonatas, M. K., Anderson, W. & Evangelista, I. 1967: The contribution of altered synapses in the senile plaque: an electron microscopic study in Alzheimer's dementia. *Journal of neuropathology and experimental neurology* **26**, 25-39.
- Gordon, I., Genis, I., Grauer, E., Schayek, E. & Michaelson, D. M. 1996a: Biochemical and cognitive studies of Apolipoprotein E deficient mice. *Molecular and chemical neuropathology* **28**, 97-103.
- Gordon, I., Genis, I., Grauer, E., Schayek, E. & Michaelson, D. M. 1996b: Biochemical and cognitive studies of apolipoprotein-E-deficient mice. *Molecular and Chemical Neuropathology* **28**, 97-103.
- Gordon, I., Grauer, E., Genis, I., Schayek, E. & Michaelson, D. M. 1995: Memory Deficits and Cholinergic Impairments in Apolipoprotein E- Deficient Mice. *Neuroscience Letters* **199**, 1-4.
- Grabowski, T. J. & Damasio, A. R. 1997: Definition, clinical features and neuroanatomical basis of dementia. In: The neuropathology of dementia (Esiri, M. M. & Morris, J. H. eds). Cambridge University Press, Cambridge. pp. 1-14.
- Grandes, P. & Streit, P. 1991: Effect of Perforant Path Lesion On Pattern of Glutamate-Like Immunoreactivity in Rat Dentate Gyrus. *Neuroscience* **41**, 391-400.
- Gundersen, V., Chaudhry, F. A., Bjaalie, J. G., Fonnum, F., Ottersen, O. P. & Storm Mathisen, J. 1998: Synaptic vesicular localization and exocytosis of L-aspartate in excitatory nerve terminals: A quantitative immunogold analysis in rat hippocampus. *Journal of Neuroscience* **18**, 6059-6070.
- Hamos, J. E., De Gennaro, L. J. & Drachman, D. A. 1989: Synaptic loss in Alzheimer's disease and other dementias. *Neurology* **39**, 355-361.
- Hampson, R. E., Simeral, J. D. & Deadwyler, S. A. 1999: Distribution of spatial and non spatial information in the dorsal hippocampus. *Nature* **402**, 610-614.
- Han, S. H., Einstein, G., Weisgraber, K. H., Strittmatter, W. J., Saunders, A. M., Pericakvance, M., Roses, A. D. & Schmechel, D. E. 1994a: Apolipoprotein-E Is Localized to the Cytoplasm of Human Cortical- Neurons - a Light and Electron-Microscopic Study. *Journal of Neuropathology and Experimental Neurology* **53**, 535-544.
- Han, S. H., Hulette, C., Saunders, A. M., Einstein, G., Pericakvance, M., Strittmatter, W. J., Roses, A. D. & Schmechel, D. E. 1994b: Apolipoprotein-e is present in hippocampal-neurons without neurofibrillary tangles in alzheimers-disease and in age-matched controls. *Experimental Neurology* **128**, 13-26.
- Handelmann, G. E., Boyles, J. K., Weisgraber, K. H., Mahley, R. W. & Pitas, R. E. 1992: Effects of apolipoprotein-e; beta-very low-density lipoproteins, and cholesterol on the extension of neurites by rabbit dorsal-root ganglion neurons invitro. *Journal Of Lipid Research* **33**, 1677-1688.
- Hardy, J. 1997: Amyloid, the presenilins and Alzheimer's disease. *Trends in Neurosciences* **20**, 154-159.
- Haug, H. 1985: Gibt es nervenzellverluste während der alterung in der menschlichen hirnrinde? *Nervenheilkunde* **4**, 103-109.

- Haug, H., Kuhl, S., Mecke, H., Sass, N. L. & Wasner, K. 1984: The significance of morphometric procedures in the investigation of age changes in cytoarchitectonic structures of human brain. *International Journal of Brain Research and Neurobiology* **25**, 353-374.
- Hayek, T., Oiknine, J., Brook, J. G. & Aviram, M. 1994: Increased Plasma and Lipoprotein Lipid-Peroxidation in Apo E- Deficient Mice. *Biochemical and Biophysical Research Communications* **201**, 1567-1574.
- Heinonen, O., Lehtovirta, M., Soininen, H., Helisalmi, S., Mannermaa, A., Sorvari, H., Kosunen, O., Paljarvi, L., Ryyanen, M. & Riekkinen, P. J. 1995a: Alzheimer Pathology of Patients Carrying Apolipoprotein-E Epsilon-4 Allele. *Neurobiology of Aging* **16**, 505-513.
- Heinonen, O., Soininen, H., Sorvari, H., Kosunen, O., Paljarvi, L., Koivisto, E. & Riekkinen, P. J. 1995b: Loss of Synaptophysin-Like Immunoreactivity in the Hippocampal- Formation Is an Early Phenomenon in Alzheimers-Disease. *Neuroscience* **64**, 375-384.
- Helkala, E. L., Koivisto, K., Hanninen, T., Vanhanen, M., Kervinen, K., Kuusisto, J., Mykkanen, L., Kesaniemi, Y. A., Laakso, M. & Riekkinen, P. 1995: The Association of Apolipoprotein-E Polymorphism With Memory - a Population-Based Study. *Neuroscience Letters* **191**, 141-144.
- Helkala, E. L., Koivisto, K., Hanninen, T., Vanhanen, M., Kervinen, K., Kuusisto, J., Mykkanen, L., Kesaniemi, Y. A., Laakso, M. & Riekkinen, P. 1996: Memory functions in human subjects with different apolipoprotein E phenotypes during a 3-year population-based follow-up study. *Neuroscience Letters* **204**, 177-180.
- Henderson, A. S., Easteal, S., Jorm, A. F., Mackinnon, A. J., Korten, A. E., Christensen, H., Croft, L. & Jacomb, P. A. 1995: Apolipoprotein-E Allele Epsilon-4, Dementia, and Cognitive Decline in a Population-Sample. *Lancet* **346**, 1387-1390.
- Hesse, C., Bogdanovic, N., Davidsson, P. & Blennow, K. 1999: A quantitative and immunohistochemical study on apolipoprotein E in grain tissue in Alzheimer's disease. *Dementia and Geriatric Cognitive Disorders* **10**, 452-459.
- Holtzman, D. M., Bales, K. R., Wu, S., Bhat, P., Parsadanian, M., Fagan, A. M., Chang, L. K., Sun, Y. L. & Paul, S. M. 1999: Expression of human apolipoprotein E reduces amyloid-beta deposition in a mouse model of Alzheimer's disease. *Journal of Clinical Investigation* **103**, R15-R21.
- Holtzman, D. M., Pitas, R. E., Kilbridge, J., Nathan, B., Mahley, R. W., Bu, G. J. & Schwartz, A. L. 1995: Low-density-lipoprotein receptor-related protein mediates apolipoprotein e-dependent neurite outgrowth in a central nervous system-derived neuronal cell-line. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **92**, 9480-9484.
- Honer, W. G., Dickson, D. W., Gleeson, J. & Davies, P. 1992a: Regional synaptic pathology in Alzheimer's disease. *Neurobiology of Aging* **13**, 375-382.
- Honer, W. G., Dickson, D. W., Gleeson, J. & Davies, P. 1992b: Regional Synaptic Pathology in Alzheimers-Disease. *Neurobiology of Aging* **13**, 375-382.
- Horsburgh, K., Fitzpatrick, M., Nilsen, M. & Nicoll, J. A. R. 1997: Marked alterations in the cellular localisation and levels of apolipoprotein E following acute subdural haematoma in rat. *Brain Research* **763**, 103-110.
- Horsburgh, K., Graham, D. I., Stewart, J. & Nicoll, J. A. R. 1999a: Influence of apolipoprotein E genotype on neuronal damage and ApoE immunoreactivity in human hippocampus following global ischemia. *Journal of Neuropathology and Experimental Neurology* **58**, 227-234.
- Horsburgh, K., Kelly, S., McCulloch, J., Higgins, G. A., Roses, A. D. & Nicoll, J. A. R. 1999b: Increased neuronal damage in apolipoprotein E-deficient mice following global ischaemia. *Neuroreport* **10**, 837-841.
- Horsburgh, K. & Nicoll, J. A. R. 1996a: Selective alterations in the cellular distribution of apolipoprotein E immunoreactivity following transient cerebral ischaemia in the rat. *Neuropathology and Applied Neurobiology* **22**, 342-349.

Horsburgh, K. & Nicoll, J. A. R. 1996b: Selective cellular alterations in amyloid precursor protein and apolipoprotein E following transient cerebral ischaemia in the rat. *Alzheimer's research* **2**, 37-42.

Howard, C. V. a. R., M.G. 1998: Estimation of reference volume using the Cavalieri method. In: Unbiased stereology. BIOS. pp. 39-54.

Huang, D. Y., Goedert, M., Jakes, R., Weisgraber, K. H., Garner, C. C., Saunders, A. M., Pericakvance, M. A., Schmechel, D. E., Roses, A. D. & Strittmatter, W. J. 1994: Isoform-specific interactions of apolipoprotein-e with the microtubule-associated protein map2c - implications for alzheimers- disease. *Neuroscience Letters* **182**, 55-58.

Huang, D. Y., Weisgraber, K. H., Goedert, M., Saunders, A. M., Roses, A. D. & Strittmatter, W. J. 1995: Apoc3 Binding to Tau Tandem Repeat-I Is Abolished By Tau Serine(262) Phosphorylation. *Neuroscience Letters* **192**, 209-212.

Hunter, A. & Stewart, M. G. 1993: Long-term increases in the numerical density of synapses in the chick lobus parolfactorius after passive avoidance training. *Brain research* **605**, 251-255.

Hyman, B. T., Gomez Isla, T., Briggs, M., Chung, H., Nichols, S., Kohout, F. & Wallace, R. 1996a: Apolipoprotein E and cognitive change in an elderly population. *Annals of Neurology* **40**, 55-66.

Hyman, B. T., Gomez Isla, T., Rebeck, G. W., Briggs, M., Chung, H. Y., West, H. L., Greenberg, S., Mui, S., Nichols, S., Wallace, R. & Growdon, J. H. 1996b: Epidemiological, clinical, and neuropathological study of apolipoprotein E genotype in Alzheimer's disease. *Annals of the New York Academy of Sciences* **802**, 1-5.

Hyman, B. T., Kromer, L. J. & Vanhoesen, G. W. 1987a: Reinnervation of the Hippocampal Perforant Pathway Zone in Alzheimers-Disease. *Annals of Neurology* **21**, 259-267.

Hyman, B. T., Van Hoesen, G. W. & Damasio, A. R. 1990: Memory-related neural systems in Alzheimer's disease: an anatomic study. *Neurology* **40**, 1721-1730.

Hyman, B. T., Van Hoesen, G. W., Kromer, L. J. & Damasio, A. R. 1986: Perforant pathway changes and the memory impairment of Alzheimer's disease. *annals of neurology* **20**, 472-481.

Hyman, B. T., Vanhoesen, G. W. & Damasio, A. R. 1987b: Alzheimers-Disease - Glutamate Depletion in the Hippocampal Perforant Pathway Zone. *Annals of Neurology* **22**, 37-40.

Hyman, B. T., Vanhoesen, G. W., Damasio, A. R. & Barnes, C. L. 1984: Alzheimers-Disease - Cell-Specific Pathology Isolates the Hippocampal-Formation. *Science* **225**, 1168-1170.

Hyman, B. T., West, H. L., Rebeck, G. W., Buldyrev, S. V., Mantegna, R. N., Ukleja, M., Havlin, S. & Stanley, H. E. 1995: Quantitative-Analysis of Senile Plaques in Alzheimer-Disease - Observation of Log-Normal Size Distribution and Molecular Epidemiology of Differences Associated With Apolipoprotein-E Genotype and Trisomy-21 (Down-Syndrome). *Proceedings of the National Academy of Sciences of the United States of America* **92**, 3586-3590.

Igbavboa, U., Avdulov, N. A., Chochina, S. V. & Wood, W. G. 1997: Transbilayer distribution of cholesterol is modified in brain synaptic plasma membranes of knockout mice deficient in the low- density lipoprotein receptor, apolipoprotein E, or both proteins. *Journal of Neurochemistry* **69**, 1661-1667.

Ignatius, M. J., Gebickeharter, P. J., Skene, J. H. P., Schilling, J. W., Weisgraber, K. H., Mahley, R. W. & Shooter, E. M. 1986: Expression of Apolipoprotein-E During Nerve Degeneration and Regeneration. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 1125-1129.

Ingram, D. K. & Jucker, M. 1999: Developing mouse models of aging: a consideration of strain differences in age-related behavioral and neural parameters. *Neurobiology of Aging* **20**, 137-145.

Ishii, K., Tamaoka, A., Mizusawa, H., Shoji, S., Ohtake, T., Fraser, P. E., Takahashi, H., Tsuji, S., Gearing, M., Mizutani, T., Yamada, S., Kato, M., StGeorgeHyslop, P. H., Mirra, S. S. & Mori, H. 1997: A beta 1-40 but not A

beta 1-42 levels in cortex correlate with apolipoprotein E epsilon 4 allele dosage in sporadic Alzheimer's disease. *Brain Research* **748**, 250-252.

Iwatsubo, T., Odaka, A., Suzuki, N., Mizusawa, H., Nukina, N. & Ihara, Y. 1994: Visualization of a-Beta-42(43) and a-Beta-40 in Senile Plaques With End-Specific a-Beta Monoclonals - Evidence That an Initially Deposited Species Is a-Beta-42(43). *Neuron* **13**, 45-53.

Jack, C. R., Petersen, R. C., O'Brien, P. C. & Tangalos, E. G. 1992: Mr-based hippocampal volumetry in the diagnosis of alzheimers-disease. *Neurology* **42**, 183-188.

Jack, C. R., Petersen, R. C., Xu, Y. C., O'Brien, P. C., Waring, S. C., Tangalos, E. G., Smith, G. E., Ivnik, R. J., Thibodeau, S. N. & Kokmen, E. 1998: Hippocampal atrophy and apolipoprotein E genotype are independently associated with Alzheimer's disease. *Annals Of Neurology* **43**, 303-310.

Jellinger, K. A. 1998: The neuropathological diagnosis of Alzheimer disease. *Journal of Neural Transmission-Supplement*, 97-118.

Ji, Z. S., Pitas, R. E. & Mahley, R. W. 1998: Differential cellular accumulation retention of apolipoprotein E mediated by cell surface heparan sulfate proteoglycans - Apolipoproteins E3 and E2 greater than E4. *Journal Of Biological Chemistry* **273**, 13452-13460.

Jobst, K. A., Smith, A. D., Szatmari, M., Esiri, M. M., Jaskowski, A., Hindley, N., McDonald, B. & Molyneux, A. J. 1994: Rapidly Progressing Atrophy of Medial Temporal-Lobe in Alzheimers- Disease. *Lancet* **343**, 829-830.

Jones, O. T., Eubanks, J. H., Earnest, J. P. & McNamee, M. G. 1988: A Minimum Number of Lipids Are Required to Support the Functional- Properties of the Nicotinic Acetylcholine-Receptor. *Biochemistry* **27**, 3733-3742.

Jordan, B. D., Relkin, N. R., Ravdin, L. D., Jacobs, A. R., Bennett, A. & Gandy, S. 1997: Apolipoprotein E epsilon 4 associated with chronic traumatic brain injury in boxing. *Jama-Journal of the American Medical Association* **278**, 136-140.

Jordan, J., Galindo, M. F., Miller, R. J., Reardon, C. A., Getz, G. S. & LaDu, M. J. 1998: Isoform-specific effect of apolipoprotein E on cell survival and beta-amyloid-induced toxicity in rat hippocampal pyramidal neuronal cultures. *Journal Of Neuroscience* **18**, 195-204.

Jucker, M. & Ingram, D. K. 1997: Murine models of brain aging and age-related neurodegenerative diseases. *Behavioural Brain Research* **85**, 1-25.

Jucker, M., Walker, L. C., Martin, L. J., Kitt, C. A., Kleinman, H. K., Ingram, D. K. & Price, D. L. 1992: Age-Associated Inclusions in Normal and Transgenic Mouse-Brain. *Science* **255**, 1443-1445.

Jucker, M., Walker, L. C., Schwarb, P., Hengemihle, J., Kuo, H., Snow, A. D., Bamert, F. & Ingram, D. K. 1994: Age-Related Deposition of Glia-Associated Fibrillar Material in Brains of C57bl/6 Mice. *Neuroscience* **60**, 875-889.

Kawas, C., Resnick, S., Morrison, A., Brookmeyer, R., Corrada, M., Zonderman, A., Bacal, C., Lingle, D. D. & Metter, E. 1997: A prospective study of estrogen replacement therapy and the risk of developing Alzheimer's disease: The Baltimore Longitudinal Study of Aging. *Neurology* **48**, 1517-1521.

Kempermann, G., Kuhn, H. G. & Gage, F. H. 1998: Experience-induced neurogenesis in the senescent dentate gyrus. *Journal of Neuroscience* **18**, 3206-3212.

Kida, E., Pluta, R., Lossinsky, A. S., Golabek, A. A., Choimiura, N. H., Wisniewski, H. M. & Mossakowski, M. J. 1995: Complete Cerebral-Ischemia With Short-Term Survival in Rat Induced By Cardiac-Arrest .2. Extracellular and Intracellular Accumulation of Apolipoprotein-E and Apolipoprotein-J in the Brain. *Brain Research* **674**, 341-346.

- Kim, D. H., Iijima, H., Goto, K., Sakai, J., Ishii, H., Kim, H. J., Suzuki, H., Kondo, H., Saeki, S. & Yamamoto, T. 1996: Human apolipoprotein E receptor 2 - A novel lipoprotein receptor of the low density lipoprotein receptor family predominantly expressed in brain. *Journal of Biological Chemistry* **271**, 8373-8380.
- King, M. V. 1991: Dimensional changes in cells and tissues during specimen preparation for the electron microscope. *Cell Biophysics* **18**, 31-55.
- Klunk, W. E. 1998: Biological markers of Alzheimer's disease. *Neurobiology of Aging* **19**, 145-147.
- Kosaka, T. & Hania, K. 1986: 3-Dimensional Structure of Astrocytes in the Rat Dentate Gyrus. *Journal of Comparative Neurology* **249**, 242-260.
- Kowal, R. C., Herz, J., Weisgraber, K. H., Mahley, R. W., Brown, M. S. & Goldstein, J. L. 1990: Opposing Effects of Apolipoprotein-E and Apolipoprotein-C On Lipoprotein Binding to Low-Density-Lipoprotein Receptor-Related Protein. *Journal of Biological Chemistry* **265**, 10771-10779.
- Krieger, M. & Herz, J. 1994: Structures and Functions of Multiligand Lipoprotein Receptors - Macrophage Scavenger Receptors and Ldl Receptor-Related Protein (Lrp). *Annual Review of Biochemistry* **63**, 601-637.
- Krzywkowski, P., Ghribi, O., Gagne, J., Chabot, C., Kar, S., Rochford, J., Massicotte, G. & Poirier, J. 1999: Cholinergic systems and long-term potentiation in memory-impaired apolipoprotein E-deficient mice. *Neuroscience* **92**, 1273-1286.
- Kuhn, H. G., DickinsonAnson, H. & Gage, F. H. 1996: Neurogenesis in the dentate gyrus of the adult rat: Age-related decrease of neuronal progenitor proliferation. *Journal of Neuroscience* **16**, 2027-2033.
- Kuller, L. H., Shemanski, L., Manolio, T., Haan, M., Fried, L., Bryan, N., Burke, G. L., Tracy, R. & Bhadelia, R. 1998: Relationship between ApoE, MRI findings, and cognitive function in the Cardiovascular Health Study. *Stroke* **29**, 388-398.
- Kuo, H., Ingram, D. K., Walker, L. C., Tian, M., Hengemihle, J. M. & Jucker, M. 1996: Similarities in the age-related hippocampal deposition of periodic acid-Schiff-positive granules in the senescence-accelerated mouse P8 and C57BL/6 mouse strains. *Neuroscience* **74**, 733-740.
- LaDu, M. J., Gilligan, S. M., Lukens, J. R., Cabana, V. G., Reardon, C. A., VanEldik, L. J. & Holtzman, D. M. 1998: Nascent astrocyte particles differ from lipoproteins in CSF. *Journal of Neurochemistry* **70**, 2070-2081.
- Landen, M., Thorsell, A., Wallin, A. & Blennow, K. 1996: The apolipoprotein-e allele epsilon-4 does not correlate with the number of senile plaques or neurofibrillary tangles in patients with alzheimers-disease. *Journal Of Neurology Neurosurgery And Psychiatry* **61**, 352-356.
- Laskowitz, D. T., Horshugh, K. & Roses, A. D. 1998: Apolipoprotein E and the CNS response to injury. *Journal Of Cerebral Blood Flow And Metabolism* **18**, 465-471.
- Laskowitz, D. T., Sheng, H. X., Bart, R. D., Joyner, K. A., Roses, A. D. & Warner, D. S. 1997: Apolipoprotein E-deficient mice have increased susceptibility to focal cerebral ischemia. *Journal Of Cerebral Blood Flow And Metabolism* **17**, 753-758.
- Lassman, H., Weiler, R., Fischer, P., Bancher, C., Jellinger, K., Floor, E., Danieleczyk, W., Seitelberger, F. & Winkler, H. 1992: Synaptic pathology in Alzheimer's disease: immunological data for markers of synaptic and large dense-core vesicles. *Neuroscience* **46**, 1-8.
- Lathe, R. 1996: Mice, gene targeting and behaviour: Than just genetic background - Commentary. *Trends in Neurosciences* **19**, 183-186.
- Lee, K. S., Standford, E. J., Cotman, C. W. & Lynch, G. S. 1977: Ultrastructural evidence for bouton proliferation in the partially deafferented denate gyrus of the rat. *Experimental brain research* **29**, 475-485.

- Lefranc, D., Vermersch, P., Dallongeville, J., DaemsMonpeurt, C., Petit, H. & Delacourte, A. 1996: Relevance of the quantification of apolipoprotein E in the cerebrospinal fluid in Alzheimer's disease. *Neuroscience Letters* **212**, 91-94.
- Lehtimäki, T., Pirttilä, T., Mehta, P. D., Wisniewski, H. M., Frey, H. & Nikkari, T. 1995: Apolipoprotein-E (ApoE) Polymorphism and Its Influence On ApoE Concentrations in the Cerebrospinal-Fluid in Finnish Patients With Alzheimers-Disease. *Human Genetics* **95**, 39-42.
- Lehtovirta, M., Laakso, M. P., Soininen, H., Helisalmi, S., Mannermaa, A., Helkala, E. L., Partanen, K., Ryyanen, M., Vainio, P., Hartikainen, P. & Riekinen, P. J. 1995: Volumes of Hippocampus, Amygdala and Frontal-Lobe in Alzheimer Patients With Different Apolipoprotein-E Genotypes. *Neuroscience* **67**, 65-72.
- Levy-Lahad, E., Wasco, W., Poorkaj, P., Romano, D. M., Oshima, J., Pettingell, W. H., Yu, C. E., Jondro, P. D., Schmidt, S. D., Wang, K., Crowley, A. C., Fu, Y. H., Guenette, S. Y., Galas, D., Nemens, E., Wijsman, E. M., Bird, T. D., Schellenberg, G. D. & Tanzi, R. E. 1995: Candidate Gene For the Chromosome-1 Familial Alzheimers-Disease Locus. *Science* **269**, 973-977.
- Linton, M. F., Gish, R., Hubl, S. T., Butler, E., Esquivel, C., Bry, W. I., Boyles, J. K., Wardell, M. R. & Young, S. G. 1991: Phenotypes of Apolipoprotein-B and Apolipoprotein-E After Liver- Transplantation. *Journal of Clinical Investigation* **88**, 270-281.
- Lippa, C. F., Hamos, J. E., Pulaskisalo, D., Degennaro, L. J. & Drachman, D. A. 1992: Alzheimers-disease and aging - effects on perforant pathway perikarya and synapses. *Neurobiology Of Aging* **13**, 405-411.
- Lippa, C. F., Saunders, A. M., Smith, T. W., Swearer, J. M., Drachman, D. A., Ghetti, B., Nee, L., PulaskiSalo, D., Dickson, D., Robitaille, Y., Bergeron, C., Crain, B., Benson, M. D., Farlow, M., Hyman, B. T., StGeorgeHyslop, P., Roses, A. D. & Pollen, D. A. 1996: Familial and sporadic Alzheimer's disease: Neuropathology cannot exclude a final common pathway. *Neurology* **46**, 406-412.
- Lippa, C. F., Smith, T. W., Saunders, A. M., Huette, C., PulaskiSalo, D. & Roses, A. D. 1997: Apolipoprotein E-epsilon 2 and Alzheimer's disease: Genotype influences pathologic phenotype. *Neurology* **48**, 515-519.
- Liu, C. J., Grandes, P., Matute, C., Cuenod, M. & Streit, P. 1989: Glutamate-Like Immunoreactivity Revealed in Rat Olfactory-Bulb, Hippocampus and Cerebellum By Monoclonal-Antibody and Sensitive Staining Method. *Histochemistry* **90**, 427-445.
- Lomnitski, L., Kohen, R., Chen, Y., Shohami, E., Trembovler, V., Vogel, T. & Michaelson, D. M. 1997: Reduced levels of antioxidants in brains of apolipoprotein E- deficient mice following closed head injury. *Pharmacology Biochemistry and Behavior* **56**, 669-673.
- Lomnitski, L., Oron, L., Sklan, D. & Michaelson, D. M. 1999: Distinct alterations in phospholipid metabolism in brains of apolipoprotein E-deficient mice. *Journal of Neuroscience Research* **58**, 586-592.
- Lovestone, S., Anderton, B. H., Hartley, C., Jensen, T. G. & Jorgensen, A. L. 1996: The intracellular fate of apolipoprotein E is tau dependent and apoE allele-specific. *Neuroreport* **7**, 1005-1008.
- Ma, J. Y., Brewer, H. B. & Potter, H. 1996: Alzheimer A beta neurotoxicity: Promotion by antichymotrypsin, ApoE4; Inhibition by A beta-related peptides. *Neurobiology of Aging* **17**, 773-780.
- Ma, J. Y., Yee, A., Brewer, H. B., Das, S. & Potter, H. 1994: Amyloid-Associated Proteins Alpha(1)-Antichymotrypsin and Apolipoprotein-E Promote Assembly of Alzheimer Beta-Protein Into Filaments. *Nature* **372**, 92-94.
- Mac Donald, M. P. & Overmier, J. B. 1998: Present imperfect: a critical review of animal models of the mnemonic impairments in Alzheimer's disease. *Neuroscience and biobehavioral reviews* **22**, 99-120.
- Mac Gowan, S. H., Wilcock, G. K. & Scott, M. 1998: Effect of gender and apolipoprotein E genotype on response to anticholinesterase therapy in Alzheimer's disease. *International journal of geriatric psychiatry* **13**, 625-630.



- Madeira, M. D., Cadeteleite, A., Andrade, J. P. & Paulabarbosa, M. M. 1991: Effects of Hypothyroidism Upon the Granular Layer of the Dentate Gyrus in Male and Female-Adult Rats - a Morphometric Study. *Journal of Comparative Neurology* **314**, 171-186.
- Mahley, R. W. 1983: Development of Accelerated Atherosclerosis - Concepts Derived From Cell Biology and Animal-Model Studies. *Archives of Pathology & Laboratory Medicine* **107**, 393-399.
- Mahley, R. W. 1988: Apolipoprotein-c - cholesterol transport protein with expanding role in cell biology. *Science* **240**, 622-630.
- Mahley, R. W. & Huang, Y. D. 1999: Apolipoprotein E: from atherosclerosis to Alzheimer's disease and beyond. *Current Opinion in Lipidology* **10**, 207-217.
- Mahley, R. W., Ji, Z. S., Brecht, W. J., Miranda, R. D. & He, D. P. 1994: Role of Heparan-Sulfate Proteoglycans and the Ldl Receptor-Related Protein in Remnant Lipoprotein Metabolism. *Annals of the New York Academy of Sciences* **737**, 39-52.
- Mann, D. M. A. 1988: The Pathological Association Between Down Syndrome and Alzheimer- Disease. *Mechanisms of Ageing and Development* **43**, 99-136.
- Mann, D. M. A. 1991: The Topographic Distribution of Brain Atrophy in Alzheimers-Disease. *Acta Neuropathologica* **83**, 81-86.
- Mann, D. M. A., Iwatsubo, T., PickeringBrown, S. M., Owen, F., Saido, T. C. & Perry, R. H. 1997: Preferential deposition of amyloid beta protein (A beta) in the form A beta(40) in Alzheimer's disease is associated with a gene dosage effect of the apolipoprotein E E4 allele. *Neuroscience Letters* **221**, 81-84.
- Mann, D. M. A., Yates, P. O. & Marcyniuk, B. 1984: Changes in Nerve-Cells of the Nucleus Basalis of Meynert in Alzheimers-Disease and Their Relationship to Aging and to the Accumulation of Lipofuscin Pigment. *Mechanisms of Ageing and Development* **25**, 189-204.
- Marques, M. A., Tolar, M., Harmony, J. & Crutcher, K. 1997: Neurotoxicity of apolipoprotein E thrombin cleavage fragments: Implications for Alzheimer's disease - Reply. *Neuroreport* **8**, 1067-1068.
- Martin, L. J., Pardo, C. A., Cork, L. C. & Price, D. L. 1994: Synaptic Pathology and Glial Responses to Neuronal Injury Precede the Formation of Senile Plaques and Amyloid Deposits in the Aging Cerebral-Cortex. *American Journal of Pathology* **145**, 1358-1381.
- Masliah, E. 1995: Mechanisms of synaptic dysfunction in alzheimers-disease. *Histology And Histopathology* **10**, 509-519.
- Masliah, E. 1998: Mechanisms of synaptic pathology in Alzheimer's disease. *Journal Of Neural Transmission Supplement*, 147-158.
- Masliah, E., Armasolo, F., Veinbergs, I., Mallory, M. & Samuel, W. 1999: Cerebrolysin ameliorates performance deficits, and neuronal damage in apolipoprotein E-deficient mice. *Pharmacology Biochemistry and Behavior* **62**, 239-245.
- Masliah, E., Fagan, A. M., Terry, R. D., Deteresa, R., Mallory, M. & Gage, F. H. 1991a: Reactive Synaptogenesis Assessed By Synaptophysin Immunoreactivity Is Associated With Gap-43 in the Dentate Gyrus of the Adult-Rat. *Experimental Neurology* **113**, 131-142.
- Masliah, E., Hansen, L., Albright, T., Mallory, M. & Terry, R. D. 1991b: Immunoelectron microscopic study of synaptic pathology in alzheimers- disease. *Acta Neuropathologica* **81**, 428-433.
- Masliah, E., Honer, W. G., Mallory, M., Voigt, M., Kushner, P., Hansen, L. & Terry, R. 1994a: Topographical distribution of synaptic-associated proteins in the neuritic plaques of alzheimers-disease hippocampus. *Acta Neuropathologica* **87**, 135-142.

- Maslah, E., Mallory, M., Decrinck, T., Deteresa, R., Lamont, S., Miller, A., Terry, R. D., Carragher, B. & Ellisman, M. 1993: Reevaluation of the structural organization of neuritic plaques in alzheimers-disease. *Journal Of Neuropathology And Experimental Neurology* **52**, 619-632.
- Maslah, E., Mallory, M., Ge, N. F., Alford, M., Veinbergs, I. & Roses, A. D. 1995: Neurodegeneration in the central-nervous-system of apoe-deficient mice. *Experimental Neurology* **136**, 107-122.
- Maslah, E., Mallory, M., Hansen, L., Deteresa, R., Alford, M. & Terry, R. 1994b: Synaptic and neuritic alterations during the progression of alzheimers-disease. *Neuroscience Letters* **174**, 67-72.
- Maslah, E., Mallory, M., Veinbergs, I., Miller, A. & Samuel, W. 1996: Alterations in apolipoprotein E expression during aging and neurodegeneration. *Progress In Neurobiology* **50**, 493-503
- Maslah, E., Samuel, W., Veinbergs, I., Mallory, M., Mante, M. & Saitoh, T. 1997: Neurodegeneration and cognitive impairment in apoE-deficient mice is ameliorated by infusion of recombinant apoE. *Brain Research* **751**, 307-314.
- Maslah, E. & Terry, R. 1993: The role of synaptic proteins in the pathogenesis of disorders of the central-nervous-system. *Brain Pathology* **3**, 77-85.
- Maslah, E., Terry, R. D., Alford, M., Deteresa, R. & Hansen, L. A. 1991c: Cortical and subcortical patterns of synaptophysinlike immunoreactivity in alzheimers-disease. *American Journal Of Pathology* **138**, 235-246.
- Maslah, E., Terry, R. D., Deteresa, R. M. & Hansen, L. A. 1989: Immunohistochemical Quantification of the Synapse-Related Protein Synaptophysin in Alzheimer-Disease. *Neuroscience Letters* **103**, 234-239.
- Maslah, E., Terry, R. D., Mallory, M., Alford, M. & Hansen, L. A. 1990: Diffuse plaques do not accentuate synapse loss in alzheimers-disease. *American Journal Of Pathology* **137**, 1293-1297.
- Matthews, D. A., Cotman, C. W. & Lynch, G. 1976: An electron microscopic study of lesion-induced synaptogenesis in the dentate gyrus of the adult rat. *Brain research* **115**, 1-21.
- May, P. C., Lampertchells, M., Johnson, S. A., Poirier, J., Masters, J. N. & Finch, C. E. 1990: Dynamics of Gene-Expression For a Hippocampal Glycoprotein Elevated in Alzheimers-Disease and in Response to Experimental Lesions in Rat. *Neuron* **5**, 831-839.
- Mayhew, T. M. 1996: How to count synapses unbiasedly and efficiently at the ultrastructural level: Proposal for a standard sampling and counting protocol. *Journal of Neurocytology* **25**, 793-804.
- McEwen, B. S., Alves, S. E., Bulloch, K. & Weiland, N. G. 1997: Ovarian steroids and the brain: Implications for cognition and aging. *Neurology* **48**, S8-S15.
- McNamara, M. J., Gomez Isla, T. & Hyman, B. T. 1998: Apolipoprotein E genotype and deposits of A beta 40 and A beta 42 in Alzheimer disease. *Archives of Neurology* **55**, 1001-1004.
- Meshul, C. K., Emre, N., Nakamura, C. M., Allen, C., Donohue, M. K. & Buckman, J. F. 1999: Time-dependent changes in striatal glutamate synapses following a 6- hydroxydopamine lesion. *Neuroscience* **88**, 1-16.
- Metzger, R. E., LaDu, M. J., Pan, J. B., Getz, G. S., Frail, D. E. & Falduto, M. T. 1996: Neurons of the human frontal cortex display apolipoprotein E immunoreactivity: Implications for Alzheimer's disease. *Journal of Neuropathology and Experimental Neurology* **55**, 372-380.
- Miller, A., Alford, M., Katzman, R., Thal, L., Saitoh, T. & Maslah, E. 1994: The expression of the ApoE4 allele in Alzheimer's disease accentuates the synaptic loss and the severity of the dementia (abstract). *Annals of Neurology* **36**, 268-269.
- Miyata, M. & Smith, J. D. 1996: Apolipoprotein E allele-specific antioxidant activity and effects on cytotoxicity by oxidative insults and beta-amyloid peptides. *Nature Genetics* **14**, 55-61.

- Mizutani, T. & Kasahara, M. 1997: Hippocampal atrophy secondary to entorhinal cortical degeneration in Alzheimer-type dementia. *Neuroscience letters* **222**, 119-122.
- Morris, C. M., Massey, H. M., Benjamin, R., Leake, A., Broadbent, C., Griffiths, M., Lamb, H., Brown, A., Ince, P. G., Tyrer, S., Thompson, P., McKeith, I. G., Edwardson, J. A., Perry, R. H. & Perry, E. K. 1996a: Molecular biology of APO E alleles in Alzheimer's and non-Alzheimer's dementias. *Journal of Neural Transmission-Supplement*, 205-218.
- Morris, J. C. 1997: The challenge of characterizing normal brain aging in relation to Alzheimer's disease. *Neurobiology of Aging* **18**, 388-389.
- Morris, J. C., McKeel, D. W., Storandt, M., Rubin, E. H., Price, J. L., Grant, E. A., Ball, M. J. & Berg, L. 1991: Very Mild Alzheimers-Disease - Informant-Based Clinical, Psychometric, and Pathological Distinction From Normal Aging. *Neurology* **41**, 469-478.
- Morris, J. C., Storandt, M., McKeel, D. W., Rubin, E. H., Price, J. L., Grant, E. A. & Berg, L. 1996b: Cerebral amyloid deposition and diffuse plaques in "normal" aging: Evidence for presymptomatic and very mild Alzheimer's disease. *Neurology* **46**, 707-719.
- Morrison, J. H. & Hof, P. R. 1997: Life and death of neurons in the aging brain. *Science* **278**, 412-419.
- Morse, J. K., Dekosky, S. T. & Scheff, S. W. 1992: Neurotrophic Effects of Steroids On Lesion-Induced Growth in the Hippocampus .2. Hormone Replacement. *Experimental Neurology* **118**, 47-52.
- Morse, J. K., Scheff, S. W. & Dekosky, S. T. 1986: Gonadal-Steroids Influence Axon Sprouting in the Hippocampal Dentate Gyrus - a Sexually Dimorphic Response. *Experimental Neurology* **94**, 649-658.
- Moser, M. B. & Moser, E. I. 1998: Functional differentiation in the hippocampus. *Hippocampus* **8**, 608-619.
- Moulder, K. L., Narita, M., Chang, L. K., Bu, G. J. & Johnson, E. M. 1999: Analysis of a novel mechanism of neuronal toxicity produced by an apolipoprotein E-derived peptide. *Journal Of Neurochemistry* **72**, 1069-1080.
- Mukaetova-Ladinska, E. B., Harrington, C. R., Roth, M. & Wischik, C. M. 1997: Presence of the apolipoprotein E type e4 allele is not associated with neurofibrillary pathology or biochemical changes to Tau protein. *Dementia* **8**, 288-295.
- Myhrer, T. 1998: Adverse psychological impact, glutamatergic dysfunction, and risk factors for Alzheimer's disease. *Neuroscience and Biobehavioral Reviews* **23**, 131-139.
- Nadler, J. V., Vaca, K. W., White, W. F., Lynch, G. S. & Cotman, C. W. 1976: Aspartate and glutamate as possible transmitters of excitatory hippocampal afferents. *Nature* **260**, 538-540.
- Nagy, Z., Esiri, M. M., Jobst, K. A., Johnston, C., Litchfield, S., Sim, E. & Smith, A. D. 1995a: Influence of the Apolipoprotein-E Genotype On Amyloid Deposition and Neurofibrillary Tangle Formation in Alzheimers-Disease. *Neuroscience* **69**, 757-761.
- Nagy, Z., Esiri, M. M., Jobst, K. A., Morris, J. H., King, E. M. F., McDonald, B., Litchfield, S., Smith, A., Barnetson, L. & Smith, A. D. 1995b: Relative Roles of Plaques and Tangles in the Dementia of Alzheimers-Disease - Correlations Using 3 Sets of Neuropathological Criteria. *Dementia* **6**, 21-31.
- Nagy, Z., Jobst, K. A., Esiri, M. M., Morris, J. H., King, E. M. F., MacDonald, B., Litchfield, S., Barnetson, L. & Smith, A. D. 1996: Hippocampal pathology reflects memory deficit and brain imaging measurements in Alzheimer's disease: Clinicopathologic correlations using three sets of pathologic diagnostic criteria. *Dementia* **7**, 76-81.
- Namba, Y., Tomonaga, M., Kawasaki, H., Otomo, E. & Ikeda, K. 1991: Apolipoprotein-e immunoreactivity in cerebral amyloid deposits and neurofibrillary tangles in alzheimers-disease and kuru plaque amyloid in creutzfeldt-jakob disease. *Brain Research* **541**, 163-166.

- Nathan, B. P., Bellosta, S., Sanan, D. A., Weisgraber, K. H., Mahley, R. W. & Pitas, R. E. 1994: Differential-effects of apolipoproteins e3 and e4 on neuronal growth in-vitro. *Science* **264**, 850-852.
- Nathan, B. P., Chang, K. C., Bellosta, S., Brisch, E., Ge, N. F., Mahley, R. W. & Pitas, R. E. 1995: The inhibitory effect of apolipoprotein e4 on neurite outgrowth is associated with microtubule depolymerization. *Journal Of Biological Chemistry* **270**, 19791-19799.
- Neve, R. L. & Robakis, N. K. 1998: Alzheimer's disease: a re-examination of the amyloid hypothesis. *Trends in Neurosciences* **21**, 15-19.
- Newman, M. F., Croughwell, N. D., Blumenthal, J. A., Lowry, E., White, W. D., Spillane, W., Davis, R. D., Glower, D. D., Smith, L. R., Mahanna, E. P. & Reves, J. G. 1995: Predictors of Cognitive Decline After Cardiac Operation. *Annals of Thoracic Surgery* **59**, 1326-1330.
- Nicoll, J. A. R., Roberts, G. W. & Graham, D. I. 1995: Apolipoprotein E E4 allele is associated with deposition of amyloid beta-protein following head injury. *Nature Medicine* **1**, 135-137.
- Nicolle, M. M., Gallagher, M. & McKinney, M. 1999: No loss of synaptic proteins in the hippocampus of aged, behaviorally impaired rats. *Neurobiology of Aging* **20**, 343-348.
- Nitsch, R. M., Blusztajn, J. K., Pittas, A. G., Slack, B. E., Growdon, J. H. & Wurtman, R. J. 1992: Evidence For a Membrane Defect in Alzheimer-Disease Brain. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 1671-1675.
- Ohm, T. G., Kirca, M., Bohl, J., Scharnagl, H., Gross, W. & Marz, W. 1995: Apolipoprotein-E Polymorphism Influences Not Only Cerebral Senile Plaque Load But Also Alzheimer-Type Neurofibrillary Tangle Formation. *Neuroscience* **66**, 583-587.
- Ohm, T. G., Scharnagl, H., Marz, W. & Bohl, J. 1999: Apolipoprotein E isoforms and the development of low and high Braak stages of Alzheimer's disease-related lesions. *Acta Neuropathologica* **98**, 273-280.
- Oitzl, M. S., Mulder, M., Lucassen, P. J., Havekes, L. M., Grootendorst, J. & deKloet, E. R. 1997: Severe learning deficits in apolipoprotein E knockout mice in a water maze task. *Brain Research* **752**, 189-196.
- Ottersen, O. P. 1989: Quantitative Electron-Microscopic Immunocytochemistry of Neuroactive Amino-Acids. *Anatomy and Embryology* **180**, 1-15.
- Ottersen, O. P. & Storm Mathisen, J. 1984: Glutamate-Containing and Gaba-Containing Neurons in the Mouse and Rat-Brain. As Demonstrated With a New Immunocytochemical Technique. *Journal of Comparative Neurology* **229**, 374-392.
- Ottersen, O. P. & Storm Mathisen, J. 1985: Different Neuronal Localization of Aspartate-Like and Glutamate-Like Immunoreactivities in the Hippocampus of Rat, Guinea-Pig and Senegalese Baboon (Papio-Papio). With a Note On the Distribution of Gamma-Aminobutyrate. *Neuroscience* **16**, 589 et seq.
- Owen, E. H., Logue, S. F., Rasmussen, D. L. & Wehner, J. M. 1997: Assessment of learning by the Morris water task and fear conditioning in inbred mouse strains and F-1 hybrids: Implications of genetic background for single gene mutations and quantitative trait loci analyses. *Neuroscience* **80**, 1087-1099.
- Oyama, F., Shimada, H., Oyama, R. & Ihara, Y. 1995: Apolipoprotein-E Genotype, Alzheimers Pathologies and Related Gene- Expression in the Aged Population. *Molecular Brain Research* **29**, 92-98.
- Page, K. J., Hollister, R. D. & Hyman, B. T. 1998: Dissociation of apolipoprotein and apolipoprotein receptor response to lesion in the rat brain: An in situ hybridization study. *Neuroscience* **85**, 1161-1171.
- Parducz, A. & Garciasegara, L. M. 1993: Sexual Differences in the Synaptic Connectivity in the Rat Dentate Gyrus. *Neuroscience Letters* **161**, 53-56.

- Parker, C. A., Callahan, M. J., Lipinski, W. J., Bisgaier, C. L. & Walker, L. C. 1996: Effects of aging on glial and neural markers in Apolipoprotein E-deficient mice. *Soc. for Neurosc. Abstr.* **22**, 207.
- Patel, S. N. & Stewart, M. G. 1988: Changes in the number and structure of dendritic spines 25 hours after passive avoidance training in the domestic chick, *Gallus Domesticus*. *Brain research* **449**, 34-46.
- Payami, H., Montee, K. R., Kaye, J. A., Wijsman, E. M., Bird, T., Yu, C. E., Heston, L. L. & Schellenberg, G. D. 1995: The apolipoprotein-e e4 allele and sex-specific risk of alzheimers- disease. *Jama Journal Of The American Medical Association* **273**, 374-374.
- Payami, H., Zarepari, S., Montee, K. R., Sexton, G. J., Kaye, J. A., Bird, T. D., Yu, C. E., Wijsman, E. M., Heston, L. L., Litt, M. & Schellenberg, G. D. 1996: Gender difference in Apolipoprotein E-associated risk for familial Alzheimer disease: A possible clue to the higher incidence of Alzheimer disease in women. *American Journal of Human Genetics* **58**, 803-811.
- Pearson, R. C. A., Esiri, M. M., Hiorns, R. W., Wilcock, G. K. & Powell, T. P. S. 1985: Anatomical Correlates of the Distribution of the Pathological-Changes in the Neocortex in Alzheimer-Disease. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 4531-4534.
- Perry, E. K., Gibson, P. H. & Blessed, G. 1977: Neurotransmitter enzyme abnormalities in senile dementia. *Journal of neurological sciences* **34**, 247-265.
- Peters, A., Rosene, D. L., Moss, M. B., Kemper, T. L., Abraham, C. R., Tigges, J. & Albert, M. S. 1996: Neurobiological bases of age-related cognitive decline in the rhesus monkey. *Journal of Neuropathology and Experimental Neurology* **55**, 861-874.
- Piedrahita, J. A., Zhang, S. H., Hagaman, J. R., Oliver, P. M. & Maeda, N. 1992: Generation of Mice Carrying a Mutant Apolipoprotein-E Gene Inactivated By Gene Targeting in Embryonic Stem-Cells. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 4471-4475.
- Pirttila, T., Soininen, H., Mehta, P. D., Heinonen, O., Lehtimäki, T., Bogdanovic, N., Paljarvi, L., Kim, K. S., Kosunen, O., Winblad, B., Riekkinen, P. & Wisniewski, H. M. 1997: Apolipoprotein E genotype and amyloid load in Alzheimer disease and control brains. *Neurobiology of Aging* **18**, 121-127.
- Pitas, R. E. 1996: Microtubule formation and neurite extension are blocked by apolipoprotein E4. *Seminars in Cell & Developmental Biology* **7**, 725-731.
- Pitas, R. E., Boyles, J. K., Lee, S. H., Hui, D. & Weisgraber, K. H. 1987: Lipoproteins and Their Receptors in the Central-Nervous-System - Characterization of the Lipoproteins in Cerebrospinal-Fluid and Identification of Apolipoprotein-B.E(Ldl) Receptors in the Brain. *Journal of Biological Chemistry* **262**, 14352-14360.
- Poirier, J. 1994: Apolipoprotein-e in animal-models of cns injury and in alzheimers- disease. *Trends In Neurosciences* **17**, 525-530.
- Poirier, J., Baccichet, A., Dea, D. & Gauthier, S. 1993a: Cholesterol-synthesis and lipoprotein reuptake during synaptic remodeling in hippocampus in adult-rats. *Neuroscience* **55**, 81-90.
- Poirier, J., Davignon, J., Bouthillier, D., Kogan, S., Bertrand, P. & Gauthier, S. 1993b: Apolipoprotein-E Polymorphism and Alzheimers-Disease. *Lancet* **342**, 697-699.
- Poirier, J., Delisle, M. C., Quirion, R., Aubert, I., Farlow, M., Lahiri, D., Hui, S., Bertrand, P., Nalbantoglu, J., Gillfix, B. M. & Gauthier, S. 1995: Apolipoprotein E4 allele as a predictor of cholinergic deficits and treatment outcome in Alzheimer disease. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **92**, 12260-12264.
- Poirier, J., Hess, M., May, P. C. & Finch, C. E. 1991: Astrocytic apolipoprotein-e messenger-rna and glap messenger-rna in hippocampus after entorhinal cortex lesioning. *Molecular Brain Research* **11**, 97-106.

- Poirier, J. & Seigny, P. 1998: Apolipoprotein E4, cholinergic integrity and the pharmacogenetics of Alzheimer's disease. *Journal Of Neural Transmission Supplement*, 199-207.
- Popko, B., Goodrum, J. F., Bouldin, T. W., Zhang, S. H. & Maeda, N. 1993: Nerve Regeneration Occurs in the Absence of Apolipoprotein-E in Mice. *Journal of Neurochemistry* **60**, 1155-1158.
- Price, D. L., Tanzi, R. E., Borchelt, D. R. & Sisodia, S. S. 1998: Alzheimer's disease: Genetic studies and transgenic models. *Annual Review of Genetics* **32**, 461-493.
- Price, J. L. 1993: The Relationship Between Tangle and Plaque-Formation During Healthy Aging and Mild Dementia. *Neurobiology of Aging* **14**, 661-663.
- Price, J. L., Davis, P. B., Morris, J. C. & White, D. L. 1991: The Distribution of Tangles, Plaques and Related Immunohistochemical Markers in Healthy Aging and Alzheimers-Disease. *Neurobiology of Aging* **12**, 295-312.
- Procter, A. W., Palmer, A. M., Francis, P. T., Lowe, S. L., Neary, D., Murphy, E., Doshi, R. & Bowen, D. M. 1988: Evidence of Glutamatergic Denervation and Possible Abnormal- Metabolism in Alzheimers-Disease. *Journal of Neurochemistry* **50**, 790-802.
- Raber, J., Wong, D., Buttini, M., Orth, M., Bellosta, S., Pitas, R. E., Mahley, R. W. & Mucke, L. 1998: Isoform-specific effects of human apolipoprotein E on brain function revealed in ApoE knockout mice: Increased susceptibility of females. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **95**, 10914-10919.
- Rajavashisth, T. B., Kaptein, J. S., Reue, K. L. & Lusis, A. J. 1985: Evolution of Apolipoprotein-E - Mouse Sequence and Evidence For an 11-Nucleotide Ancestral Unit. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 8085-8089.
- Rapp, P. R. & Amaral, D. G. 1992: Individual-Differences in the Cognitive and Neurobiological Consequences of Normal Aging. *Trends in Neurosciences* **15**, 340-345.
- Rapp, P. R. & Gallagher, M. 1996: Preserved neuron number in the hippocampus of aged rats with spatial learning deficits. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 9926-9930.
- Rapp, P. R., Stack, E. C. & Gallagher, M. 1999: Morphometric studies of the aged hippocampus: I. Volumetric analysis in behaviorally characterized rats. *Journal of Comparative Neurology* **403**, 459-470.
- Rasmussen, T., Schliemann, T., Sorensen, J. C., Zimmer, J. & West, M. J. 1996: Memory-impaired aged rats - no loss of principal hippocampal and subicular neurons. *Neurobiology Of Aging* **17**, 143-147.
- Rebeck, G. W., Harr, S. D., Strickland, D. K. & Hyman, B. T. 1995: Multiple, Diverse Senile Plaque-Associated Proteins Are Ligands of an Apolipoprotein-E Receptor, the Alpha(2)-Macroglobulin Receptor Low- Density-Lipoprotein Receptor-Related Protein. *Annals of Neurology* **37**, 211-217.
- Rebeck, G. W., Reiter, J. S., Strickland, D. K. & Hyman, B. T. 1993: Apolipoprotein-E in Sporadic Alzheimers-Disease - Allelic Variation and Receptor Interactions. *Neuron* **11**, 575-580.
- Reed, T., Carmelli, D., Swan, G. E., Breitner, J. C. S., Welsh, K. A., Jarvik, G. P., Deeb, S. & Auwerx, J. 1994: Lower Cognitive Performance in Normal Older Adult Male Twins Carrying the Apolipoprotein-E Epsilon-4 Allele. *Archives of Neurology* **51**, 1189-1192.
- Rempel Clower, N. L., Zola, S. M., Squire, L. R. & Amaral, D. G. 1996: Three cases of enduring memory impairment after bilateral damage limited to the hippocampal formation. *Journal of Neuroscience* **16**, 5233-5255.
- Roses, A. D., Gilbert, J., Xu, P. T., Sullivan, P., Popko, B., Burkhart, D. S., ChristianRothrock, T., Saunders, A. M., Maeda, N. & Schmechel, D. E. 1998: Cis-acting human ApoE tissue expression element is associated with human pattern of intraneuronal ApoE in transgenic mice. *Neurobiology Of Aging* **19**, S53-S58.

- Roses, A. D., Saunders, A. M., Corder, E. H., Risch, N. J., Haines, J. L., Pericakvance, M. A., Han, S. H., Einstein, G., Hulette, C., Schmechel, D. E., Goedert, M., Jakes, R., Dong, L. M., Weisgraber, K. H., Holsti, M., Huang, D. & Strittmatter, W. J. 1994: Apolipoprotein-e and alzheimer-disease. *Neurobiology Of Aging* **15**, S150.
- Rothstein, J. D., Martin, L., Levey, A. I., Dykeshoberg, M., Jin, L., Wu, D., Nash, N. & Kuncel, R. W. 1994: Localization of Neuronal and Glial Glutamate Transporters. *Neuron* **13**, 713-725.
- Roullet, P. & Lassalle, J. M. 1995: Radial Maze-Learning Using Exclusively Distant Visual Cues Reveals Learners and Nonlearners Among Inbred Mouse Strains. *Physiology & Behavior* **58**, 1189-1195.
- Rusakov, D. A., Davies, H. A., Harrison, E., Diana, G., RichterLevin, G., Bliss, T. & Stewart, M. G. 1997: Ultrastructural synaptic correlates of spatial learning in rat hippocampus. *Neuroscience* **80**, 69-77.
- Rusakov, D. A., Harrison, E. & Stewart, M. G. 1998: Synapses in hippocampus occupy only 1-2% of cell membranes and are spaced less than half-micron apart: a quantitative ultrastructural analysis with discussion of physiological implications. *Neuropharmacology* **37**, 513-521.
- Rusakov, D. A., Stewart, M. G., Davies, H. A. & Harrison, E. 1993: Spatial rearrangement of the vesicle apparatus in forebrain synapses of chicks 30 min after passive avoidance training. *Neuroscience letters* **154**, 13-16.
- Rusakov, D. A., Stewart, M. G., Davies, H. A. & Harrison, E. 1995: Population trends in the fine spatial reorganization of synaptic elements in forebrain regions of chicks 0.5 and 24 hours after passive avoidance training. *Neuroscience* **66**, 291-307.
- Saito, S., Kobayashi, S., Ohashi, Y., Igarashi, M., Komiya, Y. & Ando, S. 1994: Decreased Synaptic Density in Aged Brains and Its Prevention By Rearing Under Enriched Environment As Revealed By Synaptophysin Contents. *Journal of Neuroscience Research* **39**, 57-62.
- Salehi, A., Martinez, V. G. & Swaab, D. F. 1998: A sex difference and no effect of ApoE type on the amount of cytoskeletal alterations in the nucleus basalis of Meynert in Alzheimer's disease. *Neurobiology Of Aging* **19**, 505-510.
- Samuel, W., Masliah, E., Hill, L. R., Butters, N. & Terry, R. 1994: Hippocampal connectivity and alzheimers dementia - effects of synapse loss and tangle frequency in a 2-component model. *Neurology* **44**, 2081-2088.
- Saunders, A. M., Schmechel, D., Breitner, J. C. S., Benson, M. D., Brown, W. T., Goldfarb, L., Goldgaber, D., Manwaring, M. G., Szymanski, M. H., McCown, N., Dole, K. C., Schmechel, D. E., Strittmatter, W. J., Pericakvance, M. A. & Roses, A. D. 1993a: Apolipoprotein-E-Epsilon-4 Allele Distributions in Late-Onset Alzheimers-Disease and in Other Amyloid-Forming Diseases. *Lancet* **342**, 710-711.
- Saunders, A. M., Strittmatter, W. J., Schmechel, D., Georgehyslop, P. H. S., Pericakvance, M. A., Joo, S. H., Rosi, B. L., Gusella, J. F., Crappermaclachlan, D. R., Alberts, M. J., Hulette, C., Crain, B., Goldgaber, D. & Roses, A. D. 1993b: Association of Apolipoprotein-E Allele Epsilon-4 With Late-Onset Familial and Sporadic Alzheimers-Disease. *Neurology* **43**, 1467-1472.
- Scheff, S. W., Dekosky, S. T. & Price, D. A. 1990: Quantitative Assessment of Cortical Synaptic Density in Alzheimers- Disease. *Neurobiology of Aging* **11**, 29-37.
- Scheff, S. W. & Price, D. A. 1993: Synapse Loss in the Temporal-Lobe in Alzheimers-Disease. *Annals of Neurology* **33**, 190-199.
- Scheff, S. W. & Price, D. A. 1998: Synaptic density in the inner molecular layer of the hippocampal dentate gyrus in Alzheimer disease. *Journal of Neuropathology and Experimental Neurology* **57**, 1146-1153.
- Scheff, S. W., Sparks, D. L. & Price, D. A. 1996: Quantitative assessment of synaptic density in the outer molecular layer of the hippocampal dentate gyrus in Alzheimer's disease. *Dementia* **7**, 226-232.
- Schmechel, D. E., Saunders, A. M., Strittmatter, W. J., Crain, B. J., Hulette, C. M., Joo, S. H., Pericakvance, M. A., Goldgaber, D. & Roses, A. D. 1993: Increased Amyloid Beta-Peptide Deposition in Cerebral-Cortex As a

- Consequence of Apolipoprotein-E Genotype in Late-Onset Alzheimer- Disease. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 9649-9653.
- Scott, B. L., Welch, K., deSerrano, V., Moss, N. C., Roses, A. D. & Strittmatter, W. J. 1998: Human apolipoprotein E accelerates microtubule polymerization in vitro. *Neuroscience Letters* **245**, 105-108.
- Scoville, W. B. & Milner, B. 1957: Loss of recent memory after bilateral hippocampal lesions. *Journal of neurology and neurosurgical psychiatry* **20**, 11-21.
- Selkoe, D. J. 1997: Neuroscience - Alzheimer's disease: Genotypes, phenotype, and treatments. *Science* **275**, 630-631.
- Sheng, H. X., Laskowitz, D. T., Bennett, E., Schmechel, D. E., Bart, R. D., Saunders, A. M., Pearlstein, R. D., Roses, A. D. & Warner, D. S. 1998: Apolipoprotein E isoform-specific differences in outcome from focal ischemia in transgenic mice. *Journal Of Cerebral Blood Flow And Metabolism* **18**, 361-366.
- Sheng, H. X., Laskowitz, D. T., Mackensen, G. B., Kudo, M., Pearlstein, R. D. & Warner, D. S. 1999: Apolipoprotein E deficiency worsens outcome from global cerebral ischemia in the mouse. *Stroke* **30**, 1118-1123.
- Sherrington, R., Rogaev, E. I., Liang, Y., Rogaeva, E. A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., Tsuda, T., Mar, L., Foncin, J. F., Bruni, A. C., Montesi, M. P., Sorbi, S., Rainero, I., Pinessi, L., Nee, L., Chumakov, I., Pollen, D., Brookes, A., Sanssou, P., Polinsky, R. J., Wasco, W., Dasilva, H. A. R., Haines, J. L., Pericakvance, M. A., Tanzi, R. E., Roses, A. D., Fraser, P. E., Rommens, J. M. & StGeorgehyslop, P. H. 1995: Cloning of a Gene Bearing Missense Mutations in Early-Onset Familial Alzheimers-Disease. *Nature* **375**, 754-760.
- Shore, V. G. & Shore, B. 1973: Heterogeneity of human plasma very low density lipoproteins. Separation of species differing in protein components. *Biochemistry* **12**, 502-507.
- Shupliakov, O., Atwood, H. L., Ottersen, O. P., Storm Mathisen, J. & Brodin, L. 1995: Presynaptic Glutamate Levels in Tonic and Phasic Motor Axons Correlate With Properties of Synaptic Release. *Journal of Neuroscience* **15**, 7168-7180.
- Shupliakov, O., Brodin, L., Cullheim, S., Ottersen, O. P. & Storm Mathisen, J. 1992: Immunogold Quantification of Glutamate in 2 Types of Excitatory Synapse With Different Firing Patterns. *Journal of Neuroscience* **12**, 3789-3803.
- Shupliakov, O., Ottersen, O. P., Storm Mathisen, J. & Brodin, L. 1997: Glial and neuronal glutamine pools at glutamatergic synapses with distinct properties. *Neuroscience* **77**, 1201-1212.
- Sidman, R. L., Angevine, J. B. & Pierce, E. T. 1971: Atlas of the Mouse Brain and Spinal Cord. Harvard University Press, Cambridge.
- Silver, L. M. 1995: Reproduction and breeding. In: Mouse genetics, concepts and applications, Vol. 4. Oxford University Press. pp. 62-75.
- Simic, G., Gnjidic, M. & Kostovic, I. 1998: Cytoskeletal changes as an alternative view on pathogenesis of Alzheimer's disease. *Periodicum Biologorum* **100**, 165-173.
- Simic, G., Kostovic, I., Winblad, B. & Bogdanovic, N. 1997: Volume and number of neurons of the human hippocampal formation in normal aging and Alzheimer's disease. *Journal of comparative neurology* **379**, 482-494.
- Sisodia, S. S., Koo, E. H., Beyreuther, K., Unterbeck, A. & Price, D. L. 1990: Evidence That Beta-Amyloid Protein in Alzheimers-Disease Is Not Derived By Normal Processing. *Science* **248**, 492-495.
- Smith, J. D., Sikes, J. & Levin, J. A. 1998: Human apolipoprotein E allele-specific brain expressing transgenic mice. *Neurobiology Of Aging* **19**, 407-413.



- Smith, M. A., Kalaria, R. N. & Perry, G. 1993: Alpha-1-Trypsin Immunoreactivity in Alzheimers-Disease. *Biochemical and Biophysical Research Communications* **193**, 579-584.
- Smith, T. D., Calhoun, H. E. & Rapp, P. R. 1999: Circuit and morphological specificity of synaptic change in the aged hippocampal formation. *Neurobiology of Aging* **20**, 357-358.
- Soininen, H. S. & Riekkinen, P. J. 1996: Apolipoprotein-e, memory and alzheimers-disease. *Trends In Neurosciences* **19**, 224-228.
- Squire, L. R. & Zola, S. M. 1996: Structure and function of declarative and nondeclarative memory systems. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 13515-13522.
- Sterio, D. C. 1984: The unbiased estimation of the number and size of arbitrary particles using the disector. *Journal of Microscopy* **134**, 127-136.
- Stern, Y., Brandt, J., Albert, M., Jacobs, D. M., Liu, X. H., Bell, K., Marder, K., Sano, M., Albert, S., Castenada, C. D., Bylsma, F., Tycko, B. & Mayeux, R. 1997: The absence of an apolipoprotein epsilon 4 allele is associated with a more aggressive form of Alzheimer's disease. *Annals Of Neurology* **41**, 615-620.
- Stewart, J. E., Skinner, E. R. & Best, P. V. 1998: Receptor binding of an apolipoprotein E-rich subfraction of high density lipoprotein to rat and human brain membranes. *International Journal of Biochemistry & Cell Biology* **30**, 407-415.
- Stewart, M. G. & Rusakov, D. A. 1995: Morphological changes associated with stages of memory formation in the chick following passive avoidance training. *Behavioural brain research* **66**, 21-28.
- Stirling, J. W. & Graff, P. S. 1995: Antigen Unmasking For Immunoelectron Microscopy - Labeling Is Improved By Treating With Sodium Ethoxide or Sodium Metaperiodate, Then Heating On Retrieval Medium. *Journal of Histochemistry & Cytochemistry* **43**, 115-123.
- Stone, D. J., Rozovsky, I., Morgan, T. E., Anderson, C. P. & Finch, C. E. 1998: Increased synaptic sprouting in response to estrogen via an apolipoprotein E-dependent mechanism: Implications for Alzheimer's disease. *Journal of Neuroscience* **18**, 3180-3185.
- Stone, D. J., Rozovsky, I., Morgan, T. E., Anderson, C. P., Hajian, H. & Finch, C. E. 1997: Astrocytes and microglia respond to estrogen with increased apoE mRNA in vivo and in vitro. *Experimental Neurology* **143**, 313-318.
- Storm Mathisen, J., Leknes, A. K., Bore, A. T., Vaaland, J. L., Edminson, P., Haug, F. M. S. & Ottersen, O. P. 1983: 1st Visualization of Glutamate and Gaba in Neurons By Immunocytochemistry. *Nature* **301**, 517-520.
- Strittmatter, W. J., Saunders, A. M., Goedert, M., Weisgraber, K. H., Dong, L. M., Jakes, R., Huang, D. Y., Pericakvance, M., Schmechel, D. & Roses, A. D. 1994: Isoform-specific interactions of apolipoprotein-e with microtubule- associated protein tau - implications for alzheimer-disease. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **91**, 11183-11186.
- Strittmatter, W. J., Saunders, A. M., Schmechel, D., Pericakvance, M., Enghild, J., Salvesen, G. S. & Roses, A. D. 1993a: Apolipoprotein-E - High-Avidity Binding to Beta-Amyloid and Increased Frequency of Type-4 Allele in Late-Onset Familial Alzheimer-Disease. *Proceedings of the National Academy of Sciences of the United States of America* **90**, 1977-1981.
- Strittmatter, W. J., Weisgraber, K. H., Huang, D. Y., Dong, L. M., Salvesen, G. S., Pericakvance, M., Schmechel, D., Saunders, A. M., Goldgaber, D. & Roses, A. D. 1993b: Binding of human apolipoprotein-e to synthetic amyloid-beta peptide - isoform-specific effects and implications for late-onset alzheimer- disease. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **90**, 8098-8102.
- Sun, Y. L., Wu, S., Bu, G. J., Onifade, M. K., Patel, S. N., LaDu, M. J., Fagan, A. M. & Holtzman, D. M. 1998: Glial fibrillary acidic protein-apolipoprotein E (apoE) transgenic mice: Astrocyte-specific expression and differing biological effects of astrocyte-secreted apoE3 and apoE4 lipoproteins. *Journal of Neuroscience* **18**, 3261-3272.

- Svensson, A. L., Warpman, U., HellstromLindahl, E., Bogdanovic, N., Lannfelt, L. & Nordberg, A. 1997: Nicotinic receptors, muscarinic receptors and choline acetyltransferase activity in the temporal cortex of Alzheimer patients with differing apolipoprotein E genotypes. *Neuroscience Letters* **232**, 37-40.
- Sze, C. I., Troncoso, J. C., Kawas, C., Mouton, P., Price, D. L. & Martin, L. J. 1997: Loss of the presynaptic vesicle protein synaptophysin in hippocampus correlates with cognitive decline in Alzheimer disease. *Journal Of Neuropathology And Experimental Neurology* **56**, 933-944.
- Talbot, C., Lendon, C., Craddock, N., Shears, S., Morris, J. C. & Goate, A. 1994: Protection Against Alzheimers-Disease With Apoe Epsilon-2. *Lancet* **343**, 1432-1433.
- Tanapat, P., Hastings, N. B., Reeves, A. J. & Gould, E. 1999: Estrogen stimulates a transient increase in the number of new neurons in the dentate gyrus of the adult female rat. *Journal of Neuroscience* **19**, 5792-5801.
- Tang, M. X., Jacobs, D., Stern, Y., Marder, K., Schofield, P., Gurland, B., Andrews, H. & Mayeux, R. 1996: Effect of oestrogen during menopause on risk and age at onset of Alzheimer's disease. *Lancet* **348**, 429-432.
- Teasdale, G. M., Nicoll, J. A. R., Murray, G. & Fiddes, M. 1997: Association of apolipoprotein E polymorphism with outcome after head injury. *Lancet* **350**, 1069-1071.
- Teng, E. & Squire, L. R. 1999: Memory for places learned long ago is intact after hippocampal damage. *Nature* **400**, 675-677.
- Terrisse, L., Poirier, J., Bertrand, P., Merched, A., Visvikis, S., Siest, G., Milne, R. & Rassart, E. 1998: Increased levels of apolipoprotein D in cerebrospinal fluid and hippocampus of Alzheimer's patients. *Journal Of Neurochemistry* **71**, 1643-1650.
- Terry, R. D., Masliah, E. & Lawrence, A. H. 1994: Structural basis of the cognitive alterations in Alzheimer's disease. In: Alzheimer's disease (Terry, R. D., Katzman, R. & Bick, K. L. eds). Raven Press, New York. pp. 179-196.
- Terry, R. D., Masliah, E., Salmon, D. P., Butters, N., DeTeresa, R., Hill, R., Hansen, L. A. & Katzman, R. 1991: Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Annals of Neurology* **30**, 572-580.
- Teter, B., HarrisWhite, M. E., Frautschy, S. A. & Cole, G. M. 1999a: Role of apolipoprotein E and estrogen in mossy fiber sprouting in hippocampal slice cultures. *Neuroscience* **91**, 1009-1016.
- Teter, B., Xu, P. T., Gilbert, J. R., Roses, A. D., Galasko, D. & Cole, G. M. 1999b: Human apolipoprotein E isoform-specific differences in neuronal sprouting in organotypic hippocampal culture. *Journal of Neurochemistry* **73**, 2613-2616.
- Teyler, T. J. & Discenna, P. 1984: The Topological Anatomy of the Hippocampus - a Clue to Its Function. *Brain Research Bulletin* **12**, 711-719.
- Tiedge, H. & Brosius, J. 1996: Translational machinery in dendrites of hippocampal neurons in culture. *Journal of Neuroscience* **16**, 7171-7181.
- Tigges, J., Herndon, J. G. & Rosene, D. L. 1995: Mild Age-Related-Changes in the Dentate Gyrus of Adult Rhesus-Monkeys. *Acta Anatomica* **153**, 39-48.
- Tigges, J., Herndon, J. G. & Rosene, D. L. 1996: Preservation into old age of synaptic number and size in the supragranular layer of the dentate gyrus in rhesus monkeys. *Acta Anatomica* **157**, 63-72.
- Tohgi, H., Takahashi, S., Kato, E., Homma, A., Niina, R., Sasaki, K., Yonezawa, H. & Sasaki, M. 1997: Reduced size of right hippocampus in 39- to 80-year-old normal subjects carrying the apolipoprotein E epsilon 4 allele. *Neuroscience Letters* **236**, 21-24.

- Tolar, M., Marques, M. A., Harmony, J. A. K. & Crutcher, K. A. 1997: Neurotoxicity of the 22 kDa thrombin-cleavage fragment of apolipoprotein E and related synthetic peptides is receptor-mediated. *Journal of Neuroscience* **17**, 5678-5686.
- Uchihara, T., Duyckaerts, C., He, Y., Kobayashi, K., Scilhean, D., Amouyel, P. & Hauw, J. J. 1995: Apoe immunoreactivity and microglial cells in alzheimers-disease brain. *Neuroscience Letters* **195**, 5-8.
- Underwood, E. E. 1970: Quantitative stereology. Addison-Wesley, Reading, MA.
- Uylings, H. B. M., Vaneden, C. G. & Hofman, M. A. 1986: Morphometry of Size Volume Variables and Comparison of Their Bivariate Relations in the Nervous-System Under Different Conditions. *Journal of Neuroscience Methods* **18**, 19-37.
- Vangool, D., Destrooper, B., Vanleuven, F., Triau, E. & Dom, R. 1993: Alpha-2-Macroglobulin Expression in Neuritic-Type Plaques in Patients With Alzheimers-Disease. *Neurobiology of Aging* **14**, 233-237.
- Veinbergs, I., Mallory, M., Mante, M., Rockenstein, E., Gilbert, J. R. & Masliah, E. 1999: Differential neurotrophic effects of apolipoprotein E in aged transgenic mice. *Neuroscience Letters* **265**, 218-222.
- Veinbergs, I. & Masliah, E. 1999: Synaptic alterations in apolipoprotein E knockout mice. *Neuroscience* **91**, 401-403.
- Wahlund, L. O., Julin, P., Lannfelt, L., Lindqvist, J. & Svensson, L. 1999: Inheritance of the ApoE epsilon 4 allele increases the rate of brain atrophy in dementia patients. *Dementia and Geriatric Cognitive Disorders* **10**, 262-268.
- Walker, L. C. 1991: Animal models of cerebral amyloidosis. *Bulletin of clinical neurosciences* **56**, 86-96.
- Wasco, W. & Tanzi, R. E. 1995: Molecular genetics of amyloid and apolipoprotein E in Alzheimer's disease. In: *Neurobiology of Alzheimer's disease* (Dawbarn, D. & Allen, S. J. eds). Bios Scientific, Oxford. pp. 51-76.
- Weisgraber, K. H., Innerarity, T. L. & Mahley, R. W. 1982: Abnormal lipoprotein receptor binding activity of the human apolipoprotein E due to cysteine-arginine interchange at a single site. *Journal of biological chemistry* **257**, 2518-2521.
- Weisgraber, K. H. & Mahley, R. W. 1996: Human apolipoprotein E: The Alzheimer's disease connection. *Faseb Journal* **10**, 1485-1494.
- Wenzel, H. J., Buckmaster, P. S., Anderson, N. L., Wenzel, M. E. & Schwartzkroin, P. A. 1997: Ultrastructural localization of neurotransmitter immunoreactivity in mossy cell axons and their synaptic targets in the rat dentate gyrus. *Hippocampus* **7**, 559-570.
- West, H. L., Rebeck, G. W. & Hyman, B. T. 1994a: Frequency of the Apolipoprotein-E Epsilon-2 Allele Is Diminished in Sporadic Alzheimer-Disease. *Neuroscience Letters* **175**, 46-48.
- West, M. J. 1993: Regionally Specific Loss of Neurons in the Aging Human Hippocampus. *Neurobiology of Aging* **14**, 287-293.
- West, M. J. 1999: Unbiased stereology? Reply. *Trends in Neurosciences* **22**, 346-347.
- West, M. J., Coleman, P. D., Flood, D. G. & Troncoso, J. C. 1994b: Differences in the pattern of hippocampal neuronal loss in normal ageing and Alzheimer's disease. *The Lancet* **344**, 769-772.
- Whitehouse, P. J., Price, D. L., Struble, R. G., Clark, A. W., Coyle, J. T. & Delong, M. R. 1982: Alzheimers-Disease and Senile Dementia - Loss of Neurons in the Basal Forebrain. *Science* **215**, 1237-1239.
- Wimer, R. E. & Wimer, C. 1985: 3 Sex Dimorphisms in the Granule Cell Layer of the Hippocampus in House Mice. *Brain Research* **328**, 105-109.

- Wisniewski, T. & Frangione, B. 1992: Apolipoprotein-E - a Pathological Chaperone Protein in Patients With Cerebral and Systemic Amyloid. *Neuroscience Letters* **135**, 235-238.
- Wood, E. R., Dudchenko, P. A. & Eichenbaum, H. 1999: The global record of memory in hippocampal neuronal activity. *Nature* **397**, 613-616.
- Woolley, C. S., Gould, E., Frankfurt, M. & McEwen, B. S. 1990: Naturally-Occurring Fluctuation in Dendritic Spine Density On Adult Hippocampal Pyramidal Neurons. *Journal of Neuroscience* **10**, 4035-4039.
- Woolley, C. S. & McEwen, B. S. 1992: Estradiol Mediates Fluctuation in Hippocampal Synapse Density During the Estrous-Cycle in the Adult-Rat. *Journal of Neuroscience* **12**, 2549-2554.
- Xu, H. X., Gouras, G. K., Greenfield, J. P., Vincent, B., Naslund, J., Mazzarelli, L., Fried, G., Jovanovic, J. N., Seeger, M., Relkin, N. R., Liao, F., Checler, F., Buxbaum, J. D., Chait, B. T., Thinakaran, G., Sisodia, S. S., Wang, R., Greengard, P. & Gandy, S. 1998a: Estrogen reduces neuronal generation of Alzheimer beta-amyloid peptides. *Nature Medicine* **4**, 447-451.
- Xu, P. T., Gilbert, J. R., Qiu, H. L., Ervin, J., RothrockChristian, T. R., Hulette, C. & Schmechel, D. E. 1999a: Specific regional transcription of apolipoprotein E in human brain neurons. *American Journal of Pathology* **154**, 601-611.
- Xu, P. T., Gilbert, J. R., Qiu, H. L., RothrockChristian, T., Settles, D. L., Roses, A. D. & Schmechel, D. E. 1998b: Regionally specific neuronal expression of human APOE gene in transgenic mice. *Neuroscience Letters* **246**, 65-68.
- Xu, P. T., Schmechel, D., Popko, B., Rothrock, T., Qiu, H. L., Pufky, J., Maeda, N., Saunders, A. M., Roses, A. D. & Gilbert, J. R. 1995: Generation of human apolipoprotein-e isoform-specific transgenic mice. *Annals Of Neurology* **38**, 290.
- Xu, P. T., Schmechel, D., Qiu, H. L., Herbstreith, M., RothrockChristian, T., Eyster, M., Roses, A. D. & Gilbert, J. R. 1999b: Sialylated human apolipoprotein E (apoE(s)) is preferentially associated with neuron-enriched cultures from APOE transgenic mice. *Neurobiology Of Disease* **6**, 63-75.
- Xu, P. T., Schmechel, D., RothrockChristian, T., Burkhart, D. S., Qiu, H. L., Popko, B., Sullivan, P., Maeda, N., Saunders, A. M., Roses, A. D. & Gilbert, J. R. 1996: Human apolipoprotein E2, E3, and E4 isoform-specific transgenic mice: Human-like pattern of glial and neuronal immunoreactivity in central nervous system not observed in wild-type mice. *Neurobiology Of Disease* **3**, 229-245.
- Yaffe, K., Cauley, J., Sands, L. & Browner, W. 1997: Apolipoprotein E phenotype and cognitive decline in a prospective study of elderly community women. *Archives of Neurology* **54**, 1110-1114.
- Yamada, T., Kondo, A., Takamatsu, J., Tateishi, J. & Goto, I. 1995: Apolipoprotein-E Messenger-Rna in the Brains of Patients With Alzheimers-Disease. *Journal of the Neurological Sciences* **129**, 56-61.
- Yamaguchi, H., Ishiguro, K., Sugihara, S., Nakazato, Y., Kawarabayashi, T., Sun, X. Y. & Hirai, S. 1994: Presence of Apolipoprotein-E On Extracellular Neurofibrillary Tangles and On Meningeal Blood-Vessels Precedes the Alzheimer Beta-Amyloid Deposition. *Acta Neuropathologica* **88**, 413-419.
- Yamauchi, K., Tozuka, M., Nakabayashi, T., Sugano, M., Hidaka, H., Kondo, Y. & Katsuyama, T. 1999: Apolipoprotein E in cerebrospinal fluid: Relation to phenotype and plasma apolipoprotein E concentrations. *Clinical Chemistry* **45**, 497-504.
- Yang, D. S., Small, D. H., Seydel, U., Smith, J. D., Hallmayer, J., Gandy, S. E. & Martins, R. N. 1999: Apolipoprotein E promotes the binding and uptake of beta-amyloid into Chinese hamster ovary cells in an isoform-specific manner. *Neuroscience* **90**, 1217-1226.
- Yasuda, M., Mori, E., Kitagaki, H., Yamashita, H., Hirono, N., Shimada, K., Maeda, K. & Tanaka, C. 1998: Apolipoprotein E epsilon 4 allele and whole brain atrophy in late- onset Alzheimer's disease. *American Journal Of Psychiatry* **155**, 779-784.

- Wisniewski, T. & Frangione, B. 1992: Apolipoprotein-E - a Pathological Chaperone Protein in Patients With Cerebral and Systemic Amyloid. *Neuroscience Letters* **135**, 235-238.
- Wood, E. R., Dudchenko, P. A. & Eichenbaum, H. 1999: The global record of memory in hippocampal neuronal activity. *Nature* **397**, 613-616.
- Woolley, C. S., Gould, E., Frankfurt, M. & McEwen, B. S. 1990: Naturally-Occurring Fluctuation in Dendritic Spine Density On Adult Hippocampal Pyramidal Neurons. *Journal of Neuroscience* **10**, 4035-4039.
- Woolley, C. S. & McEwen, B. S. 1992: Estradiol Mediates Fluctuation in Hippocampal Synapse Density During the Estrous-Cycle in the Adult-Rat. *Journal of Neuroscience* **12**, 2549-2554.
- Xu, H. X., Gouras, G. K., Greenfield, J. P., Vincent, B., Naslund, J., Mazzarelli, L., Fried, G., Jovanovic, J. N., Seeger, M., Relkin, N. R., Liao, F., Checler, F., Buxbaum, J. D., Chait, B. T., Thinakaran, G., Sisodia, S. S., Wang, R., Greengard, P. & Gandy, S. 1998a: Estrogen reduces neuronal generation of Alzheimer beta-amyloid peptides. *Nature Medicine* **4**, 447-451.
- Xu, P. T., Gilbert, J. R., Qiu, H. L., Ervin, J., RothrockChristian, T. R., Huette, C. & Schmechel, D. E. 1999a: Specific regional transcription of apolipoprotein E in human brain neurons. *American Journal of Pathology* **154**, 601-611.
- Xu, P. T., Gilbert, J. R., Qiu, H. L., RothrockChristian, T., Settles, D. L., Roses, A. D. & Schmechel, D. E. 1998b: Regionally specific neuronal expression of human APOE gene in transgenic mice. *Neuroscience Letters* **246**, 65-68.
- Xu, P. T., Schmechel, D., Popko, B., Rothrock, T., Qiu, H. L., Pufky, J., Maeda, N., Saunders, A. M., Roses, A. D. & Gilbert, J. R. 1995: Generation of human apolipoprotein-e isoform-specific transgenic mice. *Annals Of Neurology* **38**, 290.
- Xu, P. T., Schmechel, D., Qiu, H. L., Herbstreith, M., RothrockChristian, T., Eyster, M., Roses, A. D. & Gilbert, J. R. 1999b: Sialylated human apolipoprotein E (apoE(s)) is preferentially associated with neuron-enriched cultures from APOE transgenic mice. *Neurobiology Of Disease* **6**, 63-75.
- Xu, P. T., Schmechel, D., RothrockChristian, T., Burkhardt, D. S., Qiu, H. L., Popko, B., Sullivan, P., Maeda, N., Saunders, A. M., Roses, A. D. & Gilbert, J. R. 1996: Human apolipoprotein E2, E3, and E4 isoform-specific transgenic mice: Human-like pattern of glial and neuronal immunoreactivity in central nervous system not observed in wild-type mice. *Neurobiology Of Disease* **3**, 229-245.
- Yaffe, K., Cauley, J., Sands, L. & Browner, W. 1997: Apolipoprotein E phenotype and cognitive decline in a prospective study of elderly community women. *Archives of Neurology* **54**, 1110-1114.
- Yamada, T., Kondo, A., Takamatsu, J., Tateishi, J. & Goto, I. 1995: Apolipoprotein-E Messenger-Rna in the Brains of Patients With Alzheimers-Disease. *Journal of the Neurological Sciences* **129**, 56-61.
- Yamaguchi, H., Ishiguro, K., Sugihara, S., Nakazato, Y., Kawarabayashi, T., Sun, X. Y. & Hirai, S. 1994: Presence of Apolipoprotein-E On Extracellular Neurofibrillary Tangles and On Meningeal Blood-Vessels Precedes the Alzheimer Beta-Amyloid Deposition. *Acta Neuropathologica* **88**, 413-419.
- Yamauchi, K., Tozuka, M., Nakabayashi, T., Sugano, M., Hidaka, H., Kondo, Y. & Katsuyama, T. 1999: Apolipoprotein E in cerebrospinal fluid: Relation to phenotype and plasma apolipoprotein E concentrations. *Clinical Chemistry* **45**, 497-504.
- Yang, D. S., Small, D. H., Seydel, U., Smith, J. D., Hallmayer, J., Gandy, S. E. & Martins, R. N. 1999: Apolipoprotein E promotes the binding and uptake of beta-amyloid into Chinese hamster ovary cells in an isoform-specific manner. *Neuroscience* **90**, 1217-1226.
- Yasuda, M., Mori, E., Kitagaki, H., Yamashita, H., Hirono, N., Shimada, K., Maeda, K. & Tanaka, C. 1998: Apolipoprotein E epsilon 4 allele and whole brain atrophy in late- onset Alzheimer's disease. *American Journal Of Psychiatry* **155**, 779-784.

Zannis, V. I., Kardassis, D. & Zanni, E. E. 1993: Genetic Mutations Affecting Human Lipoproteins, Their Receptors, and Their Enzymes. *Advances in Human Genetics* **21**, 145-379.

Zarow, C. & Victoroff, J. 1998: Increased apolipoprotein E mRNA in the hippocampus in Alzheimer disease and in rats after entorhinal cortex lesioning. *Experimental Neurology* **149**, 79-86.

## **Chapter 6   Appendix**

# Appendix

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## *Rinsing saline solution for perfusion*

### **0.9% sodium chloride solution**

For 100ml

- Add 0.9g NaCl to 100ml of distilled water

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### **0.1M Phosphate buffer (PB) pH 7.4**

Prepare stock solution of 0.2M PB

For 100ml

- Add 0.497g of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  to 2.328g of  $\text{Na}_2\text{HPO}_4$  in 100ml of distilled water

Add equal amount of distilled water to 0.2M PB to obtain 0.1M PB

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## *Fixative solution for EM*

### **2% Paraformaldehyde 2% Glutaraldehyde 0.1M Phosphate buffer**

For 100ml

- Add 2g paraformaldehyde to 35ml distilled water and 0.5ml of approximately 1M NaOH (made fresh each time by dissolving 5 pellets of NaOH in approximately 5 ml of distilled water)
- Heat the paraformaldehyde solution in a fume cupboard to 60°C until the paraformaldehyde dissolves
- Cool and add 8ml of EM grade 25% glutaraldehyde
- Make up to 50ml with distilled water
- Make up to 100ml with 0.2M PB pH 7.4
- Filter before use in animals

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## *Staining solution for volume estimation using Cavalieri 's method*

### **0.01% toluidene blue solution**

For 100ml

Add 0.01g of toluidene blue powder to 100ml of 0.1M PB



**Epon resin (medium hardness)**

For 38ml

-Warm to 60°C for at least 10 minutes:

glass cylinder

mixing container (plastic disposable bottle)

stock bottles of Epon resin (Agar 100), DDSA and MNA

-Pour 20ml of Agar 100 resin (24g) into cylinder, add 16ml of DDSA (16g) and 8ml of MNA (10g) and pour into mixing container. Mix by rotating gently by hand for 1-2 minutes, then place on the rotator for 10 minutes.

-Add 1.3ml of BDMA accelerator (3%). Mix as before for 1-2 minutes by hand, then place on the rotator for 10 minutes.

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*Tissue post-fixation, dehydration and embedding for electron microscopy*

**Post-fixation of fixed tissue:**

Buffer wash	2x 10 min
Post-fixation in 1%Osmium tetroxyde	1 hour
Buffer wash	10 min

**Dehydration and infiltration in resin**

Both are performed with a Lynx microscopy tissue processor:

1. 30% acetone	10 min	20°C	agitation
2. 50% acetone	20 min	20°C	agitation
3. 70% acetone	20 min	20°C	agitation
4. 90% acetone	20 min	20°C	agitation
5. 100% acetone	20 min	20°C	agitation
6. 100% acetone	20 min	20°C	agitation
7. 100% acetone (molecular sieve)	20 min	20°C	agitation
8. 50:50 Epon: acetone (mol. sieve)	3 h	20°C	agitation
9. 60: 40 Epon: acetone (mol. sieve)	3 h	20°C	agitation
10. 70: 30 Epon: acetone (mol. sieve)	3 h	20°C	agitation
11. 80: 20 Epon: acetone (mol. sieve)	16 h	20°C	agitation
12. Epon	6 h	20°C	agitation

**Embedding**

Embed in fresh Epon resin in polythene capsules with paper labels with codes written in pencil

**Polymerisation**

Polymerise at 60C for 24-48h

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*Preparation of solution for support films for ultrathin sections*

**1.5% pioloform in Analar chloroform**

Add 100ml of Analar chloroform to 1.5g of pioloform

Leave overnight to dissolve

Adjust concentration with Analar chloroform to obtain films of silver-gold interference colour.

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*EM staining of ultrathin sections*

Staining is performed in LKB ultrastainer with Leica commercial stains:

Wetting step

Ultrastain 1: Uranyl acetate	50 min	35°C
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Ultrastain 2: Lead citrate	10 min	20°C
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Wash step in de-ionised water

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*Staining solution for light microscopy*

**1% Toluidene blue 5% Borax**

For 100ml

Add 1g of toluidene blue to 5g of borax in 100ml of distilled water

Stir and heat to dissolve

Filter

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*Solutions for Post-embedding immunocytochemistry*

Buffer for immunocytochemistry

**PBSA pH 7.4**

For 1 litre

Weigh: 8g of NaCl

0.2g of KCl

1.15g of  $\text{Na}_2\text{HPO}_4$

0.2g of  $\text{KH}_2\text{PO}_4$

Add to 1 litre of distilled water

Check pH is 7.4

Blocking agents

**5% BSA/PBSA**

for 20ml

Add 1g bovine serum albumin (BSA) to 20ml PBSA

Leave overnight to dissolve

Microfilter before use

**5% BSA/2%NGS/PBSA**

for 20ml

Add 400µl of normal goat serum (NGS) to 20ml of microfiltered 5%BSA/PBSA

Buffer for antigen unmasking procedure

**0.01M sodium citrate buffer pH6**

For 100ml

-Prepare stock solutions A and B (100ml each):

-solution A = 0.2M  $\text{Na}_2\text{HPO}_4$

add 2.83g of  $\text{Na}_2\text{HPO}_4$  to 100ml of distilled water

-solution B = 0.1M citric acid

add 2.1g of citric acid to 100ml of distilled water

-Add 63.1ml of solution A to 36.9ml of solution B